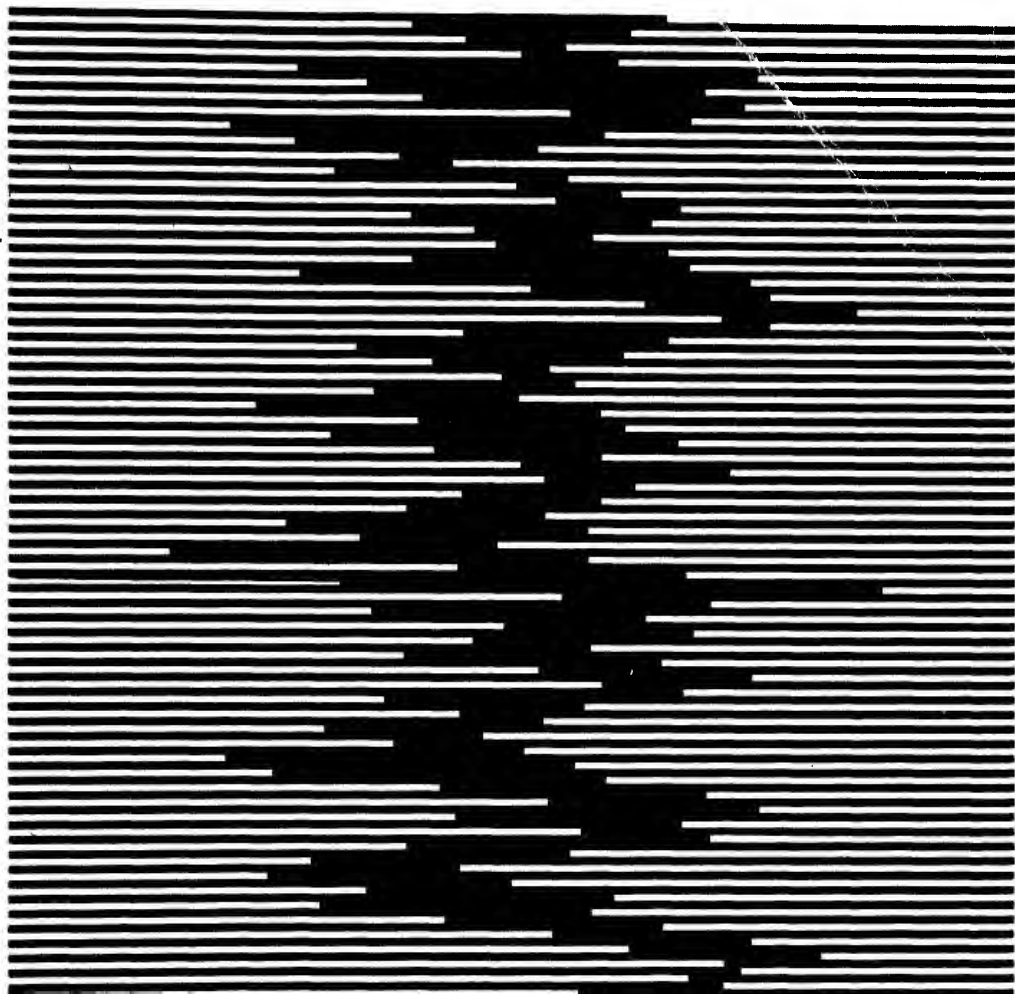


The Health Effects of Nitrate, Nitrite, and *N*-Nitroso Compounds



The Health Effects Nitrate, Nitrite, and *N*-Nitroso Compounds

Part 1 of a 2-Part Study by the Committee
Nitrite and Alternative Curing Agents in
Assembly of Life Sciences

UNIVERSITY
RARIES



whose members are drawn from the Councils of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine. The members of the committee responsible for the report were chosen for their special competences and with regard for appropriate balance.

This report has been reviewed by a group other than the authors according to procedures approved by a Report Review Committee consisting of members of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine.

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In September 1980 the Department of Agriculture and the Food and Drug Administration entered into a contract with the National Academy of Sciences to examine the health effects of dietary nitrate and nitrite and to evaluate possible alternatives to nitrite added to food as a preservative. Accordingly, the Committee on Nitrite and Alternative Curing Agents in Food was established within the Assembly of Life Sciences of the National Academy of Sciences/National Research Council. The committee was charged with the task of reviewing scientific literature pertaining to these subjects and preparing two reports. In this volume, its first report, the committee has attempted to assess the health risks associated with overall exposure to nitrate, nitrite, and N-nitroso compounds, placing emphasis on the risks resulting from natural and added nitrate and nitrite in food and the utility of nitrite added to food. In the second report, it will review the status of research and future prospects for developing feasible alternatives to the use of nitrite as a preservative.

A special effort was made to ensure that the collective knowledge of the committee encompassed all the types of expertise needed to conduct a study of this scope. The resultant multidisciplinary committee includes the biomedical expertise that was needed to evaluate the toxicological and carcinogenic significance of exposures to food additives and environmental chemicals, the metabolism and pharmacokinetics of xenobiotic compounds, and the practicality, antimicrobial efficacy, and utility of food additives.

A broad search was conducted to gather the information needed by the committee during its study. This effort went beyond a review of the vast scientific literature to include requests for information from scientists not on the committee, federal agency officials, and consultants from the food industry and trade associations. Consultants were also invited to make oral presentations or to prepare papers for consideration by the committee. In January 1981, a widely advertised public meeting was held in an attempt to ensure that all those who wished to contribute material to the committee had the opportunity to do so.

The committee recognizes that the subject of its study is of great interest to the public, which is concerned about the safety of the food supply; to the food industry, which must provide a safe and economical product while earning a fair commercial return; and to the regulatory agencies, which are responsible for monitoring products

realized that it would be helpful to view the contribution of nitrite as one component of the overall risk that might be posed by exposure to N-nitroso compounds.

The scientific questions addressed by the committee were complex. Among the most important were the following: Of what relevance to public health is the addition of nitrite when nitrate and nitrite are present naturally in foods? In what manner and to what degree do naturally occurring and added nitrate and nitrite contribute to the formation of N-nitroso compounds? What is the impact on human health resulting from the overall exposure to these compounds? For what purposes are nitrate and nitrite added to foods? How precisely can one define the resulting effects? What are the relative risks of added nitrite compared to the risks of not adding it? Are there suitable alternatives? If so, are their effects on health understood?

To address these complex, but discrete questions, the committee formed from among its members two closely interacting subgroups. Each was given the primary responsibility for the initial analysis of the evidence pertaining to one of the two major subject areas--the risks and utility of nitrate and nitrite in food or the status of research on alternative curing agents. However, each subgroup contributed to the work of its counterpart through discussions and shared writing efforts. The committee as a whole reviewed this report, and resulting comments have been incorporated into the text.

Judgments balancing economic considerations and health effects were deliberately omitted from this report because the committee believed that such considerations are not solely scientific in nature but, rather, overlap into the public policy arena.

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MACLYN McCARTY
Chairman
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EXECUTIVE SUMMARY

HISTORICAL PERSPECTIVE

Curing salts, some of which contain nitrate and nitrite, have been used for many centuries to preserve meat. However, the intentional use of nitrate and nitrite salts to cure meat is a relatively new practice.

Since the 1900s, the U.S. Department of Agriculture (USDA) has regulated and monitored the addition of nitrate and nitrite to red-meat and poultry products. The intentional use of these compounds was originally motivated by their ability to produce a reddish-pink color in meat. Subsequently, it was discovered that nitrite inhibited the growth of certain bacteria such as putrefactive anaerobes, which cause the spoilage of meats, and Clostridium botulinum, which causes a foodborne intoxication -- botulism.

In recent years, a number of observations have led to concern about potential risks to human health resulting from the use of nitrate and nitrite. At present, primary concern is focused on the possibility of carcinogenic effects, especially since nitrite can interact with substrates such as amines or amides to produce N-nitroso compounds, which can contaminate the nitrite-preserved foods. Many of these N-nitroso compounds (which include nitrosamines) are known to cause cancer in many animal species. In mid-1978, this concern was exacerbated by the results of a 2-year feeding study in animals, which suggested that nitrite per se causes cancer. These results would have necessitated the banning of nitrite in order to comply with the Food Safety Provisions and, for some uses of nitrite, the Delaney Clause of the Food, Drug, and Cosmetics Act, which proscribes the addition of known carcinogens to foods. However, further evaluation of these data suggested that the initial conclusion may not have been justified. Not only did the effects of nitrate and nitrite on human health need to be fully assessed, their contribution to the total body burden of nitrosamines had also to be determined. Thus, in 1980 the USDA and the Food and Drug Administration (FDA) asked the National Academy of Sciences to examine the current state of knowledge concerning these issues and to assess the status of research on curing agents that can be used as alternatives to nitrite.

At present, sodium nitrite is added to most bacon at 120 mg/kg, to other pickle-cured products at 200 mg/kg, and to most comminuted (e.g., chopped or ground) cured products at 156 mg/kg.

The specific contribution of nitrite to the inhibition of potential pathogens and microorganisms that are responsible for spoilage varies with the products in which it is used and with variations in their processing, handling, and abuse (such as poor refrigeration of perishable foods).

Nitrite retards microbial spoilage of cured meats by inhibiting the growth of a variety of microorganisms, especially anaerobic and aerobic spore-forming bacteria. In association with other components in the curing salt mix, it exerts a concentration-dependent antimicrobial effect in cured products including, but not limited to, inhibition of the outgrowth of spores from Clostridium botulinum and other clostridia. However, under conditions of excessive contamination or prolonged temperature abuse, it does not indefinitely prevent such outgrowth. Thus, toxin production or spoilage may ultimately ensue.

Depending on a number of factors, including the concentration of nitrite, environmental conditions, and the type of food product, nitrite may also contribute to the control of other pathogens, including Bacillus cereus, Staphylococcus aureus, and Clostridium perfringens. However, nitrite is not generally considered to be a key factor in the control of these bacteria.

Other properties of nitrite are its ability to inhibit lipid oxidation (rancidity) in cured meats and its chemical reaction with myoglobin to produce a reddish-pink color in the muscle tissue of cured meats.

The contribution of nitrite to the flavor of cured meats varies among products, but has not been fully determined for all cured meats. It appears to contribute significantly to the flavor of pickle-cured hams and ham-based food items. However, sodium chloride is largely responsible for the "cured" flavor of some foods, especially bacon. "Flavor" characteristics may not be attributable to a specific chemical component of cured meats since the olfactory system can "fuse" individual aromas into one that is different from those of its components.

Detailed information on the utility of nitrite is provided in Chapter 3.

Chemical experiments have demonstrated that nitrate, nitrite, and some oxides of nitrogen can interact with substances such as secondary or tertiary amines or amides, resulting in the formation of N-nitroso compounds. These compounds may also be produced endogenously from the same precursors. In addition to nitrosamines, classes of N-nitroso compounds include nitrosamides (such as nitrosoureas, nitrosoguanidines, and nitrosocarbamates).

Certain agents or conditions have been shown to enhance or inhibit the nitrosation reactions that lead to the formation of N-nitroso compounds. For example, nitrosation of secondary amines by aqueous nitrous acid can be catalyzed by thiocyanate and iodide and by some phenols, thiols, and alkenes. On the other hand, nitrosation of secondary amines, amides, ureas, and guanidines by aqueous nitrous acid can be inhibited by ascorbic acid (vitamin C), α -tocopherol (vitamin E), and certain naturally occurring phenols and thiols.

Transnitrosation (the transfer of a nitroso group from a nitroso compound to an amino compound) of organic nitroso and nitro compounds can take place in vitro in simple chemical systems, but there is no information on its occurrence in foods.

The extent to which N-nitroso compounds are formed in foods is affected by the type of matrix (hydrophilic or hydrophobic), the presence of natural antioxidants, and methods of processing and cooking. Nitrogen oxides generated during the processing of foods may also produce N-nitroso compounds.

The chemistry of nitrate, nitrite, and N-nitroso compounds is discussed further in Chapter 4.

ENVIRONMENTAL DISTRIBUTION AND EXPOSURE OF HUMANS

Nitrate, Nitrite, and Nitrogen Oxides

Levels of nitrate and nitrite vary widely among foods and in the environment. In foods, they depend on a number of factors, including agricultural practices and storage conditions. These factors, combined with the limitations in analytical techniques for measuring nitrate and nitrite and in methods for the accurate determination of food consumption, make it difficult to estimate precisely the exposure of humans to these compounds. Nevertheless, the committee has made estimates of such exposures. These estimates should not be taken at face value; rather, they should be used as a guide to gain an under-

Differing lifestyles and dietary habits can lead to wide variations in the amount of nitrate ingested by different population groups. In the average diet, vegetables contribute most of the nitrate ingested (~87% of daily intake). Therefore, vegetarians may consume substantially higher amounts of nitrate than does the general population. Milk generally contains very low levels of nitrate. Under certain circumstances, however, its nitrate content may be considerably higher. For example, high concentrations may be found in milk produced by cows grazed on forage with a high nitrate content. Thus, some milk may be an important source of nitrate for infants. Other sources of exposure include nitrate-rich drinking water and fruit juices.

Of the average daily intake of dietary nitrite, ~ 39% is contributed by ingestion of cured meats, ~ 34% by baked goods and cereals, and ~ 16% by vegetables. The concentration of nitrite in these foods, especially in cured meats, varies widely, and, depending on lifestyle and dietary habits, the daily exposure to nitrite from any one source can vary from 0 to 90%. Thus, there may be considerable variation in the total daily intake of nitrite. (The total gastric nitrite load, which includes nitrite resulting from the reduction of nitrate in vivo, is discussed below in the section entitled Metabolism and Pharmacokinetics.)

Three additional factors are important for determining the significance of exposure to nitrite. First, vegetables contain inhibitors of nitrosation, such as ascorbic acid and polyphenols, and possibly catalysts that can enhance nitrosation. These tend to affect the extent of in vivo nitrosation and, thus, the synthesis of N-nitroso compounds. Second, at the time of consumption, the amount of residual nitrite remaining in cured meat products (about 10 mg/kg) may be substantially lower than the amount estimated to be added during processing. This level, and thus the exposure, will vary, depending on the product. Finally, assays for the residual nitrite content of processed meats may not necessarily indicate the amount of nitrite that is available to participate in nitrosation reactions in vivo (i.e., some forms of bound nitrite may not be measured).

Although the intake of nitrogen oxides may contribute to the daily exposure to nitrate and nitrite, the contribution from this source is relatively small for the average U.S. citizen. However, peak levels of nitrogen oxides in smog-laden cities may result in more substantial exposure. These compounds are unlikely to be of major importance in converting ingested amines via nitrite to nitrosamines in the stomach. However, much remains to be learned about the role

A number of substrates in the diet, including secondary or tertiary amines or amides, may interact with nitrite to form N-nitroso compounds. Foods, drugs, cosmetics, agricultural chemicals (e.g., pesticides), tobacco, and certain occupational settings are all significant sources of nitrosatable amines and amides. There is an enormous variation in the rate at which these compounds are nitrosated and the extent to which humans are exposed to them. In addition to exogenous sources of amines, nitrosatable amines such as dimethylamine and pyrrolidine are synthesized endogenously. There are only limited data on exogenous exposure to amines and on their endogenous production. However, evidence indicates that sufficient quantities of amines are present to participate in endogenous nitrosation reactions and that nitrosation does occur when amines and/or amides and nitrate and/or nitrite are ingested simultaneously. The key factors that determine the extent of these reactions in the stomach are the pH; the concentrations of nitrate, nitrite, nitrosatable amines and/or amides; and the rate of nitrosation of the amino compound. In addition, the presence of modifiers (catalysts and inhibitors) of nitrosation reactions will influence the extent of in vivo nitrosation.

Chapter 6 contains discussions of the environmental distribution of amines and certain modifiers of nitrosation reactions.

N-Nitroso Compounds

Analytical methods are sufficiently sensitive to measure volatile nitrosamines. Consequently, the levels of many of these compounds in various environmental sources have been determined. By comparison, very little is known about the occurrence and exposure of humans to unstable and/or nonvolatile N-nitroso compounds.

Humans may be exposed to preformed nitrosamines in the environment via inhalation, ingestion, and dermal contact. Moreover, nitrosamines and nitrosamides may be formed in the body from various precursors.

Because large quantities of nitrosamines are formed in certain occupational settings and are present in tobacco and tobacco smoke, humans may be exposed to high concentrations of these compounds from these sources. For example, maximum exposure to N-nitrosodimethylamine (NDMA) at 440 $\mu\text{g}/\text{day}$ was estimated to occur in a leather-tanning facility. In one study of airborne concentrations in the rubber industry, a maximum N-nitrosomorpholine (NMOR) level of 250 $\mu\text{g}/\text{m}^3$ was found. At this concentration, daily exposure would be 2,500 $\mu\text{g}/\text{day}$. Maximum exposure for humans to NDMA in a rock t

1.4 μg per French filter cigarette and approximately 11 μg for one small cigar. If these data are typical of an average cigarette, a pack of 20 U.S. filter cigarettes would provide an intake of ~ 17 μg . By comparison, the intake of nitrosamines from all dietary sources, including beer, has been estimated to be only 1.1 μg per day.

Assays of foodstuffs in the Netherlands and the Federal Republic of Germany have indicated that, until this year, the largest single dietary source of nitrosamines was beer. However, the concentrations of these compounds in beer have been decreased by recent modifications in the malting process. Currently, the most important sources of nitrosamines in the diet are cured meat products, especially bacon, which may contribute approximately 0.17 μg of N-nitrosopyrrolidine to the total daily intake of nitrosamines. Other important exogenous sources of nitrosamines are cosmetics, pharmaceuticals, pesticides, water, and air.

In summary, with the exception of occupational exposures, which were not considered in the above calculations, cigarette smoking contributes the greatest amount to total nitrosamine intake.

Exogenous exposure to nitrosamines is discussed further in Chapter 7.

METABOLISM AND PHARMACOKINETICS

Endogenous Exposure to Nitrate and Nitrite

The metabolism and pharmacokinetics of nitrate and nitrite vary among individuals because of differences in physiology, age, and general health. The conversion of nitrate to nitrite by bacterial reduction in the saliva is an important metabolic reaction. Certain clinical conditions, such as gastric achlorhydria (abnormally low acidity in the stomach) and urinary tract infections, can also greatly enhance the opportunity for bacterial reduction of nitrate to nitrite. However, in persons with normal gastric acidity, nitrate is converted to nitrite mainly by microflora in the saliva. Approximately 25% of ingested nitrate is reported to be recirculated into the saliva, and approximately 20% of salivary nitrate is reduced to nitrite.

The contribution of various sources to exogenous exposure to nitrate and nitrite is discussed in the section on environmental distribution and exposure of humans. However, chemical changes that occur in the saliva and upper gastrointestinal tract affect the endogenous

to the gastric nitrite load are vegetables (~72%), cured meats (~9%), baked goods and cereals (~7%), and fruits and fruit juices (~5%). These estimates do not take into consideration the contribution of drinking water, which may be the major source of nitrate intake in some areas.

Endogenous Synthesis of Nitrate

The formation of nitrate by bacteria in the large intestine (heterotrophic nitrification) had been postulated as one mechanism to account for differences in ingestion and urinary excretion of nitrate in humans. However, this conclusion appears to be erroneous since studies in germ-free rodents indicate that such reactions are not important. Moreover, the nitrate content of ingested food, water, and air may have been underestimated in the earlier studies. Recent studies suggest that mammalian tissues synthesize nitrate and that this may partially explain excess urinary nitrate excretion.

In-Vivo Nitrosation

The formation of N-nitroso compounds in vivo has been well-documented in laboratory animals. In humans, the evidence is sparse. However, one recent study showed that a noncarcinogenic nitrosamine was synthesized in a human subject following the ingestion of an amine (proline) and nitrate. In that experiment, the ingestion of a large excess of ascorbic acid or α -tocopherol effectively reduced the endogenous formation of nitrosamines. Based on the methodology used in this experiment, the committee has estimated that the amount of preformed nitrosamines in the diet of the average person is roughly equivalent to the amount formed in vivo from the intake of nitrate and nitrite. However, for special population groups, such as those ingesting high-nitrate water, the increased intake of nitrate could lead to a corresponding increase in the amount of nitrosamines formed in vivo.

In addition to chemicals such as thiocyanate, ascorbic acid, and α -tocopherol, which are capable of modifying nitrosation reactions in vivo, it has been suggested that biological factors may also play an important role in the formation of nitrosamines. For example, bacteria may modify these reactions in such organs as the achlorhydric stomach and the infected bladder, where they colonize.

Studies have demonstrated that nitrosamines require metabolic activation in order to become carcinogenic; whereas nitrosamides, including nitrosoureas, nitrosoguanidines, and nitrosocarbamates, are direct-acting carcinogens. The active chemical species alkylates deoxyribonucleic acid (DNA). The organotropic, toxic, and carcinogenic effects of nitrosamines probably result from preferential metabolism by specific organs. The primary reaction required for activation may involve an enzyme-mediated α -hydroxylation. Oxidative dealkylation appears to be necessary for the toxic and carcinogenic action of nitrosamines. The balance among adduct formation, repair mechanisms, and cellular replication may be the most important determinant of the carcinogenic process. Mechanisms for repairing the DNA alkylation damage have been demonstrated in a number of tissues, including the liver and kidney.

See Chapter 8 for additional information on the metabolism and pharmacokinetics of nitrate, nitrite, and N-nitroso compounds.

ADVERSE EFFECTS ON HEALTH

Acute Toxicity in Humans

Ingestion of sufficiently large amounts of nitrate has been shown to cause methemoglobinemia -- excessive production of abnormal hemoglobin -- primarily in infants. Information on the distribution of nitrate in foods and the exposure of the general population to nitrate indicates that such acute toxicity is likely to occur rarely in the United States and generally only in people who have consumed well water contaminated with high levels of nitrate. Nitrite-induced methemoglobinemia is even less common, but has been observed to result from ingestion of home-processed vegetables. N-Nitroso compounds are acutely toxic to humans only at levels that are much higher than those normally encountered in the environment.

Chronic Toxicity in Humans

Evidence implicating nitrate, nitrite, and N-nitroso compounds in the development of cancer in humans is largely circumstantial. Epidemiological studies have suggested a possible association between exposure to high levels of nitrate and nitrite and a high incidence of stomach and esophageal cancer. For example, a high incidence of stomach cancer was found in regions of Colombia where well water contained high concentrations of nitrate. In the Henan Province in China, there is a high incidence of esophageal cancer. The formation

also identified. Thus, it is not yet known whether nitrate or nitrite play any role in the causation of these cancers.

Chronic Toxicity in Other Species

Studies conducted to determine the carcinogenicity of nitrate and nitrite have not provided sufficient evidence to conclude that these agents are carcinogenic. Nitrite is known to be mutagenic in microbial tests, and under certain conditions it interacts with nitrosatable substances to produce N-nitroso compounds. Tests for carcinogenicity in animals provide evidence that N-nitroso compounds are likely to be carcinogenic in humans. For example, most of the approximately 300 N-nitroso compounds tested have been shown to be carcinogenic in one or more species of animals. There is a wide range in the carcinogenic potency of these compounds; the potency of some is relatively high.

Tests have also indicated the importance of enhancers and inhibitors of carcinogenicity. For example, agents that promote cell proliferation in the liver enhance hepatocarcinogenicity of certain nitrosamines. Compounds such as ascorbic acid, α -tocopherol, and other antioxidants can inhibit carcinogenicity by blocking the formation of N-nitroso compounds from nitrite and nitrosatable substances.

Many N-nitroso compounds have been shown to be mutagenic in microbial tests, either directly or with metabolic activation. Mammalian-cell-mediated mutagenicity assays may ultimately provide a quantitative indication of carcinogenic activity in animals and in humans.

The adverse effects on health are discussed further in Chapter 9.

RISK ESTIMATION

Evidence of carcinogenicity provided by well-conducted experiments in animals should be regarded as indicating a potential for carcinogenicity in humans. This is especially true when results of investigations have demonstrated carcinogenicity in more than one species -- as they have for N-nitroso compounds, which have been shown to be carcinogenic in numerous species of animals.

However, there is no completely reliable method for using data obtained from animal experiments to derive the magnitude of tissue- or organ-specific carcinogenic potency of a chemical in humans. Furthermore, reliable estimates cannot be made because of the lack

for example. There are insufficient data concerning the average and extreme levels of exposure to nitrate, nitrite, and N-nitroso compounds. There are many inadequately characterized variables that determine the extent of endogenous nitrosation. There is uncertainty about the molecular mechanisms leading to the carcinogenic effect of N-nitroso compounds and their precursors and uncertainty about the comparable ability of humans and laboratory animals to repair genotoxic damage.

There is a possibility that individuals and subgroups of the population vary in their susceptibility to the carcinogenic effects of N-nitroso compounds. Additional uncertainty is introduced by the selection of a set assumptions and mathematical models to develop risk estimates. Therefore, the committee suggests that the numerical estimates in this report be used solely as rough indicators of the relative risk to each of these population groups.

Chapter 10 contains a framework for estimating risk and some first approximations of risk estimates for exposure to nitrate, nitrite, and N-nitroso compounds. The committee wishes to emphasize that although the estimates may be valuable in providing insights into the relative risks for various population groups from exposure to these compounds, their principal value is to provide a point of departure for scientists who may later, on the basis of better data, refine the risk estimates. Therefore, although the numbers and the ranges presented in Chapter 10 provide useful information on the relativity of risks in population subgroups, they are not intended as a guide for policy formation, nor should they be perceived by the public as conclusive.

Although a reduction in exposure to nitrite is likely to reduce the risk of cancer, there is insufficient evidence to support the plausible assumption that a reduced exposure to nitrate and nitrite will lead to a directly proportional reduction in the risk to human health. There is better evidence for N-nitroso compounds: Studies of N-nitrosodimethylamine in animals indicate that a directly proportional reduction in risk could result from the reduction of exposure to N-nitroso compounds.

Nitrosamines formed endogenously from nitrite ingested in cured meats provide only a small proportion of the total exposure of the general population to nitrosamines from all sources. Thus, it does not appear that the reduction of nitrite in cured meats will lead to a major decrease in risk to humans arising from total nitrosamine exposure. However, if only dietary contributors to exposure to N-nitroso compounds are considered, the diminution in risk will be proportionally greater if nitrite were removed from cured meats.

from omitting nitrite from cured meats. It found that previous attempts to derive such estimates were based on speculation with which it did not wholly concur. It concluded that a more adequate data base must be developed before one can predict the likelihood of a product becoming toxic and, from this, the incidence of botulism.

The committee believes that the degree of protection against botulism is likely to decrease if the essential preservative uses of nitrite are substantially reduced without introducing an efficacious, but safer alternative.

RECOMMENDATIONS

1. Results of limited experiments suggest that nitrate is neither carcinogenic nor mutagenic. However, evidence from several epidemiological studies in human populations is consistent with the hypothesis that exposure to high levels of nitrate may be associated with an increased incidence of cancer of the stomach and the esophagus. Thus, the committee recommends that to confirm these preliminary findings, future epidemiological studies focus on correlating the incidence of cancer and established precursor lesions with actual exposure to nitrate, nitrite, N-nitroso compounds, nitrosatable substances, and inhibitors or enhancers of nitrosation. Where possible, exposure should also be correlated with levels of nitrate, nitrite, and N-nitroso compounds in biological fluids such as blood, saliva, or urine.

2. Evidence does not indicate that nitrite acts directly as a carcinogen in animals. However, because it is mutagenic in microbial systems and because of its implied role in the induction of esophageal and stomach cancer in humans, further testing in animals may be warranted. If such tests provide any indication of carcinogenicity, then the committee recommends that attempts be made to distinguish between the types of carcinogenic activity, i.e., activity as a complete carcinogen, cocarcinogen, or promoter.

3. Most N-nitroso compounds are carcinogenic in laboratory animals, mutagenic in microbial and mammalian test systems, and some are teratogenic in laboratory animals. Although these tests are indicative of potential carcinogenicity in humans, they are of limited value for predicting the quantitative risk to humans. The committee recommends that future carcinogenicity assays emphasize quantitative assessment of potency as well as the qualitative outcome. It also recognizes the need to characterize premalignant lesions induced by N-nitroso compounds and to develop short-term in vivo bioassays to determine their carcinogenicity.

mittee recommends that exposure of humans to these agents be reduced. Exposure to nitrite should be reduced to the extent that protection against botulism is not compromised. Additionally, the committee recommends that, with the exception of dry-cured products and fermented sausage products in which the presence of nitrate may be necessary, the use of nitrate salts in the curing process be discontinued in all meat and poultry products. Furthermore, the committee suggests that attention should be given to the feasibility of reducing the nitrate content of vegetables and drinking water and that further studies should be conducted to develop methods to reduce nitrate in vegetables while maintaining the content of ascorbic acid and other inhibitors of nitrosation.

5. The committee suggests that the sources of exposure to N-nitroso compounds in various environmental media be determined so that methods to reduce the exposure to these contaminants can be developed. Standardized analytical methods are needed to assess the total body burden of nonvolatile N-nitroso compounds. In addition, it is necessary to obtain accurate estimates of exposure to nitrate and nitrite by improving the assay procedures, especially to distinguish between free and bound nitrite, and to determine whether the residual nitrite is a true measure of nitrosating capacity.

6. The exposure of humans to amines and nitrosamines can be reduced in certain circumstances by modifying manufacturing practices that result in high levels of exposure. For example, pesticides produced as secondary and tertiary amine salts could be replaced by other formulations and certain readily nitrosated drugs could be replaced by drugs that have the same therapeutic effect but are not nitrosated. Further research should be conducted to identify amino compounds that could be nitrosated in vivo, especially those that are readily nitrosated or to which humans are extensively exposed.

7. The committee believes that additional studies are needed to increase understanding of the metabolism and pharmacokinetics of nitrate in humans. Also requiring clarification is the role of bacteria in the reduction of nitrate to nitrite and the formation of N-nitroso compounds, especially in certain clinical conditions such as gastric achlorhydria and bladder infection.

8. The nitrosation-inhibiting effects of ascorbate and other substances have been established, and this knowledge has been put to use commercially to inhibit the formation of nitrosamines in bacon. Normal dietary constituents that enhance or inhibit nitrosation should be studied further to determine the extent of their effects in the diet and in vivo. Specifically, further research is needed to determine the amount of nitrite that is destroyed in the human stomach and the extent

9. Further studies are required to determine the mechanisms whereby nitrite controls the outgrowth of C. botulinum spores. Research is also needed to determine its mechanism of action in cured meats, especially its antioxidant activity and its effect against microorganisms that are responsible for spoilage and against pathogens other than C. botulinum. Since the effect of nitrite varies considerably among products, it should be examined on a product-by-product basis.

10. Although it is not possible to estimate the potential morbidity or mortality from C. botulinum in the absence of nitrite as a curing agent in certain products, the prudent approach to protecting public health requires consideration of the possibility that certain preserved food items may be contaminated and may be abused.

11. In view of the possible but unquantified risk resulting from the use of nitrite as a curing agent, the committee recommends that the search for alternatives and alternative approaches to the use of nitrite be continued. However, no new agent or combination of agents should be substituted for nitrite until adequate testing has ensured that it does not present a hazard to human health.

CHAPTER 2

HISTORICAL PERSPECTIVE

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CHAPTER 2

HISTORICAL PERSPECTIVE

Salting has been used to preserve the aesthetic and healthful qualities of meat and fish in most civilizations for more than 3,000 years. In their review of curing practices, Binkerd and Kolari (1975) speculated that curing with salt was first practiced in saline desert or coastal regions, e.g., near the Dead Sea. They noted that desert salt contains nitrate as an impurity and that saltpeter, or nitre (potassium nitrate), or "wall saltpeter" (calcium nitrate) from cave walls were used by ancient people for their preservative quality. The earliest specific mention of the characteristic pink color of cured meat did not appear until late Roman literature (Binkerd and Kolari, 1975). References to the flavor conferred by saltpeter as part of the curing mixture were made as early as 1835 (Binkerd and Kolari, 1975), but it was not until much later that this effect was scientifically investigated (Brooks *et al.*, 1940). By the late 1800s, saltpeter was specifically recommended as an ingredient in curing recipes to promote the development of "cured color." For example, Edward Smith in 1873 observed that "Meat, when prepared by salt alone, loses its colour, but when saltpetre is added the flesh becomes a reddish color throughout, provided the action be sufficiently prolonged." (Smith, 1873, p. 35).

Failures to achieve consistent results in curing, particularly in the attainment of safety of cured products, were well documented by Kerner in Germany (1817, 1820, 1822). He studied many outbreaks of often-fatal "sausage poisonings" or "botulism" (a term derived from the Latin "botulus" for sausage) and identified the omission of nitrate from the salt mixture used to cure incriminated sausage as a common feature of the outbreaks. Van Ermengen (1897) demonstrated that the cause of botulism was a neurotoxin produced in the food by a bacterium. He identified this microorganism as an anaerobic spore-forming bacillus, which he named Bacillus botulinus. It is now known as Clostridium botulinum. The first recorded case of botulism in the United States occurred in 1899 (Center for Disease Control, 1979). Botulism is traditionally regarded as the most serious, life-threatening foodborne disease of microbial origin. It is discussed in more detail in the last section of this chapter.

in the late 19th century. Polenske (1891) reported the presence of nitrite in cured meat and in pickling solutions used for curing. He attributed this to the reduction of nitrate to nitrite by bacterial action.

Lehmann (1899) and Kisskalt (1899) independently reported that nitrite rather than nitrate conferred the typical color to cured products. Subsequently, Haldane (1901), on the basis of experiments with blood and hemoglobin, proposed that the reaction of hemoglobin with nitric oxide derived from nitrite was the chemical basis for the cured meat color. This reaction mechanism was confirmed in uncooked cured meats and sausages by Hoagland (1910).

Although many old curing mixes contained nitrate and possibly nitrite impurities, it is not known when nitrite itself was first purposefully used in curing salt. But as early as 1917, Doran received a patent in the United States for the use of nitrite in curing (Doran, 1917).

RECOGNITION OF THE ANTIMICROBIAL ACTIVITY OF NITRITE

The specific contribution of nitrite (and indirectly nitrate) to the antimicrobial effects of the curing salt mixture was not recognized until the late 1920s. Kerr et al. (1926) stated that neither nitrate nor nitrite had any preservative value, but 2 years later Lewis and Moran (1928) suggested that nitrite had antimicrobial effects. This was later confirmed by other investigators (Evans and Tanner, 1934; Tarr, 1941, 1942, 1944).

A review by Tanner (1944) indicates some of the uncertainties at that time regarding the antimicrobial actions of nitrite and the magnitude of its role in the inhibition of C. botulinum in cured meats. Steinke and Foster (1951) appear to have been the first investigators to provide definitive evidence of sodium nitrite's antibotulinal efficacy in a meat product when it is added at the levels commonly used by commercial producers today.

In the two subsequent decades, many reports on the antibotulinal activity of nitrite were published, and it became generally accepted that nitrite exerted such activity in most cured products (Foster and Duncan, 1974; Sofos et al., 1979). However, definitive evidence of the relative contribution of nitrite in controlling C. botulinum in various foods has only been obtained for certain products during the last decade (Christiansen et al., 1973, 1974, 1975; Hustad et al., 1973).

Of the nitrate and nitrite added to foods in the United States, most has traditionally been used in the production of red-meat products. The responsibility for assuring the safety of such products resides with the U.S. Department of Agriculture (USDA) as it has since 1907 when its Meat Inspection Act (P.L. 242) was passed.

Shortly thereafter, the use of potassium nitrate in curing salt mixes was sanctioned (U.S. Department of Agriculture, 1908), and sodium nitrate was permitted in 1922 (U.S. Department of Agriculture, 1922). However, the demonstration that the active "color-fixing" agent was, in fact, nitrite offered the possibility that the substitution of nitrite for nitrate could provide more consistent results in the curing process, especially in the production of cured color.

In 1923, pilot studies on the use of sodium nitrite, without nitrate(s), in the curing salt mix began under the supervision of the USDA. These studies focused predominantly on the development of cured color. The investigators concluded that sodium or potassium nitrate could be successfully replaced by sodium nitrite in the curing of meat. The quantity of sodium nitrite needed ranged from 0.25 to 1 oz per 100 lb of meat (156-625 mg/kg), depending on the type of meat and the process used. They also observed that the curing period could be shortened by the use of sodium nitrite (Kerr et al., 1926).

On the basis of these results, the USDA in 1925 authorized the use of sodium nitrite as a substitute for sodium or potassium nitrate in the curing of meat (U.S. Department of Agriculture, 1925). The agency suggested that nitrite could be substituted for nitrate at approximately one-tenth the weight of nitrate. At that time, the USDA established 200 mg/kg as a maximum allowable residual level of sodium nitrite in the meat after processing (Kerr et al., 1926; U.S. Department of Agriculture, 1925). This level was lower than the residual level resulting from the use of nitrate(s) at that time. Since no acute toxic effects had been observed to result from these residual concentrations, it was presumed that the 200 mg/kg level was safe.

Thus, these regulations permitted a "mixed" cure, i.e., one containing both nitrate and nitrite, but no residual limit was imposed on nitrate. In 1926, observations that meat processors were using more nitrate than needed led to the issuance of a regulation limiting the level of nitrate salt to 1% in the pickling solution in which meat was immersed (U.S. Department of Agriculture, 1926).

nitrite cure for sausages and comminuted meats. However, some processors continued to favor the use of a mixed cure and, in 1931, the USDA approved a mixed cure containing 0.25 oz of sodium nitrite and 2.75 oz of nitrate salt per 100 lb of meat, i.e., 156 mg/kg and 1,716 mg/kg, respectively (Tanner, 1944).

Binkerd and Kolari (1975), Cervený (1980), and Sebranek (1979) have reviewed studies that indicate a slow trend over the ensuing decades toward the use of nitrite cures. However, the levels of nitrite and nitrate, which formed the basis of the early regulations (Tanner, 1944; U.S. Department of Agriculture, 1925), were reiterated in the 1970 USDA Meat Inspection Regulations (U.S. Department of Agriculture, 1970, p. 15590). These regulations permit the addition of sodium or potassium nitrite at 2 lb per 100 gallons of pickling solution, which results in an initial concentration of approximately 200 mg/kg meat, 1 oz per 100 lb of meat in dry cure (625 mg/kg), or 0.25 oz per 100 lb in chopped meat products (156 mg/kg). Sodium or potassium nitrate were permitted at 7 lb per 100 gallons of pickle (~700 mg/kg), 3.5 oz to 100 lb of meat in dry cure (~2,200 mg/kg), or 2.75 oz per 100 lb of chopped meat (~1,700 mg/kg). The use of nitrate, nitrite, or combinations could not result in a residual of more than 200 mg/kg nitrite, calculated as sodium nitrite, in the finished product.

These regulations are in force today (Code of Federal Regulations, 1981a) for all cured meats except bacon. Subsequent regulations limiting the amount of nitrite and nitrate in bacon are discussed later in this chapter.

In the early 1960s, there were several outbreaks of botulism attributed to temperature-abused,¹ smoked fish processed without the use of nitrate or nitrite in the Great Lakes region of the United States (Center for Disease Control, 1979). Subsequently, research was conducted to establish guidelines for the use of nitrite to produce safe smoked fish products.

The addition of nitrate and nitrite to fish products is regulated by the Food and Drug Administration (FDA). These regulations currently permit up to 500 mg residual sodium nitrate per kilogram or up to 200 mg residual sodium nitrite per kilogram in smoked cured sable fish, shad, and salmon as a preservative and color fixative (Code of Federal Regulations, 1981d,e); a residual of between 100 and 200 mg sodium nitrite per kilogram in hot smoked chub to inhibit

per kilogram in smoked cured tuna as a color fixative (Code of Federal Regulations, 1981e); and up to 200 mg residual potassium nitrate per kilogram in cod roe as a curing agent (Code of Federal Regulations, 1981c).

TRENDS IN THE PRODUCTION OF CURED MEATS

Reviews of the trends in the production and distribution of cured meat products have been published by Binkerd and Kolari (1975), Cervený (1980), and Sebranek (1979). Some of these trends, especially those pertaining to refrigeration, are pertinent to the safety or spoilage of cured meats.

During the first two decades of this century, mechanical refrigeration was practiced in processing plants and warehouses, but during distribution, which was largely accomplished by horsedrawn wagons, ice or ice and salt was used for cooling. Local distribution and retail display were often unaccompanied by refrigeration, and home use of ice boxes was limited. Meat curing processes relied predominantly on heavy salting or smoking for preservation without refrigeration. Thermal processing (canning) was just beginning to be used (Anonymous, 1952; Cervený, 1980).

In the 1920s, hygiene in processing plants was improved by the adoption of solvent extraction of spices for flavorings and the use of aqueous chlorine as a sanitizing agent for food and equipment. Both served to reduce microbial contamination of products (Anonymous, 1952; Reddish, 1957; White, 1972). During this decade, motorized transportation became more widespread, but the mechanically refrigerated trucks used by ice-cream manufacturers were adopted only slowly by meat packers, most of whom continued to use ice or ice and salt for chilling. Retail outlets began to use display cases chilled by ice or ice and salt, and the use of iceboxes in private homes increased (Anonymous, 1952; Cervený, 1980).

Between 1930 and 1940, meat product distributors began to use mechanically refrigerated delivery trucks (Fowler, 1952), and mechanical refrigerators were installed in many retail stores and homes. This increased use of refrigeration had a substantial impact on the meat product industry. Milder cures could be used since refrigeration complemented preservation by curing, and meat packers began to package processed meats in consumer-sized packages. Moreover, raw materials and finished products could be collected from and distributed to larger geographical areas. Thus, many operations expanded considerably, and the number of individual producers started to decline. Canned luncheon meats, sausages, and hams were also intro-

increased in-plant packaging of sausages and sliced meats. During the war years, certain cured products, e.g., more highly salted bacon, were developed specifically for military operations when refrigeration was not readily available (Cerveny, 1980).

Shortly after 1950, oxygen-impermeable films were developed for the packaging of meat products, replacing the oxygen-permeable packaging that had been in use up to that time. The new packaging delayed color fading and spoilage of products by aerobic microorganisms. The subsequent introduction of vacuum packaging increased the expected shelf life of many perishable cured meats to 30 and up to 60 days (Cerveny, 1980), when these products were properly refrigerated. Such packaging also inhibits spoilage at higher temperatures to the extent that certain products are acceptable from a sensory viewpoint after 6 or more days of storage at 20°C to 30°C (Pivnick and Bird, 1965; Pivnick et al., 1967) -- temperatures at which the growth of C. botulinum is possible.

THE ORIGINS OF RECENT CONCERN ABOUT NITRATE AND NITRITE IN FOODS

Nitrite can react with nitrosatable substrates such as amines to produce potentially carcinogenic N-nitroso compounds such as N-nitrosamines² (see Chapters 4, 8, and 9). Thus, current concern over the use of nitrate and nitrite in foods stems not from the potential for acute toxic sequelae, but mostly from possible chronic, carcinogenic effects. The occurrence of nitrosation reactions has been known for more than 100 years (Hein, 1963; Ridd, 1961); however, simple and reliable methods to detect the reaction products at the levels present in foods exist only for the volatile nitrosamines, and these methods have only been developed recently (see Chapter 7).

Following indications that N-nitrosodimethylamine might be the cause of cirrhosis and other toxic effects in industrial workers, Barnes and Magee (1954) examined the toxicity of this compound in rats, dogs, rabbits, mice, and guinea pigs. Their results indicated that N-nitrosodimethylamine was hepatotoxic in the animals listed and that the lesions resembled those induced by liver carcinogens. In subsequent investigations, they demonstrated that it was carcinogenic in the rat (Magee and Barnes, 1956).

²Except where specified otherwise, the term nitrosamine is used to imply N-nitrosamines

and hamsters, were susceptible to at least one agent (Chapter 9).

Possible nitrosamine contamination of foods preserved with nitrite was first indicated in the early 1960's when outbreaks of hepatotoxicosis in mink and sheep were reported from Norway (Boehler, 1960, 1962). These outbreaks of liver toxicity were traced to N-nitrosodimethylamine arising from the addition of nitrite as a preservative to herring meal, the drying of the meal at high temperatures, and its use in feeds (Ender et al., 1964, 1967; Hansen, 1964; Koppang, 1964; Sakshaug et al., 1965).

Outbreaks of botulism caused by smoked fish in the early 1960s prompted research on processes using nitrite to inhibit C. botulinum in fish products. Concern about the possible production of nitrosamines through this use led to an investigation of their distribution in nitrite-treated fish by Fazio et al. (1971), who demonstrated that N-nitrosodimethylamine was present in products from marine species, such as salmon and shad, that had been treated with nitrate, nitrite, or both, at the levels then permitted.

Results of analyses performed in the late 1960s and early 1970s indicated that nitrosamines were present in a number of foods, including cured meats and cheeses. These studies were reviewed by Sebranek and Cassens (1973), Scanlan (1975), Crosby (1976), and Crosby and Sawyer (1976), who noted that several volatile nitrosamines occurred sporadically in the lower $\mu\text{g/kg}$ range in cured meat and fish products, but that N-nitrosopyrrolidine was found rather consistently in cooked bacon at concentrations ranging from 1 to 100 $\mu\text{g/kg}$.

While the use of nitrite in smoked fish and the implications of food contamination by preformed nitrosamines were being debated, the possibility that nitrosamines could be formed endogenously from the reaction of nitrite with amines in the human stomach was indicated by the experiments of Sander and coworkers (Sander, 1967, 1968; Sander and Seif, 1969; Sander et al., 1968) and by Sen et al. (1969). (See review by Mirvish, 1975.) The regulatory dilemma thus became more complex.

Responding to their mandate to ensure the wholesomeness of the food supply, the USDA and the FDA in 1970 formed a group to coordinate the activities of the two agencies and to define research needs in collaboration with the meat-curing industry and with academia. In 1971, concern about the use of nitrite was expressed to the House Intergovernmental Relations Subcommittee at hearings on nitrosamines (U.S. Department of Agriculture, 1975). In 1972, the USDA was petitioned by consumer representatives to ban or greatly reduce the

denied by the USDA, which ruled that further information was needed on the formation of nitrosamines. When the petitioners took the issue to court, the case was dismissed on procedural grounds. Thus, the denial remained in effect. In the early 1970's, the USDA, the FDA, and representatives of the meat-processing industry agreed upon and commenced research to define more precisely the need for nitrite in cured products (U.S. Department of Agriculture, 1975). This work, reported by Christiansen et al. (1973, 1974, 1975) and Hustad et al. (1973), clarified the inhibitory effect of nitrite against C. botulinum.

In Canada, Sen et al. (1973) demonstrated that nitrosamines, especially N-nitrosopiperidine, were formed in sausage-curing premixes containing nitrite and spices. This resulted from the reaction of nitrite with amino compounds in the spices. In the United States, rapid action to verify this finding resulted in the banning of such premixes (Code of Federal Regulations, 1981b).

In the early 1970s, reports of nitrosamine contamination of cured meats, especially cooked bacon, continued to accumulate (Gray, 1976; Scanlan, 1975; Sebranek and Cassens, 1973), and in 1973, the Secretary of Agriculture appointed an advisory Expert Panel on Nitrite and Nitrosamines. The panel was charged with the task of reviewing information concerning the presence of nitrosamines in foods, evaluating the significance to public health and specific problems associated with the use of nitrite in foods, and determining if there were alternative methods of processing.

In September 1974, the Expert Panel submitted a preliminary report on its review and evaluation of the literature and other pertinent information. In response, the USDA published proposals for regulations incorporating the following recommendations made by the panel (U.S. Department of Agriculture, 1975, p. 52614):

1. That use of nitrate salts in the curing process be discontinued in all meat and poultry products with two exceptions, dry-cured products and fermented sausage products....
2. That the level of nitrite salt permitted to be added for curing of meat and poultry be limited to 156 parts per million (ppm) in all processed products, with the exception of bacon and dry-cured products....

The USDA also proposed a prohibition of the addition of nitrate and nitrite to baby foods and foods for toddlers, a maximum concentration of 125 ppm (mg/kg) for sodium nitrite added to bacon, a requirement for the addition of 500 ppm (mg/kg) sodium ascorbate or erythorbate (isoascorbate) to bacon to inhibit nitrosamine formation, and a minimum level of sodium chloride when food preservation was intended. During 1976, the USDA reviewed comments received in response to its proposals, and in 1977, with the FDA, it attempted to clarify questions pertaining to the status of nitrite as a "prior sanctioned" substance used as a preservative in poultry products and as a color fixative in red meat. Concurrently, research was being conducted to find ways to reduce the nitrosamine contamination of cured products. As techniques became more sensitive and selective, they indicated that levels of nitrosamines were highest in cooked bacon (Scanlan, 1975; U.S. Department of Agriculture, 1978b).

In October 1977, the USDA published a notice in the Federal Register asking interested parties to provide data demonstrating that bacon could be produced with low levels of nitrosamines through other processing or manufacturing procedures. The original submission date of January 16, 1978 was later extended. While the Expert Panel was deliberating, endogenous synthesis of nitrate and nitrite was reported by Tannenbaum et al. (1978). Although this paper was later challenged (Archer et al., in press; Witter et al., 1979), it brought into the debate the question of the relative contribution to exposure of humans from nitrite added to foods as compared to the total exposure from nitrite, nitrate, and nitrosamines from all sources.

In its final report, issued in February 1978, the Expert Panel recommended ingoing and residual levels of nitrite and ascorbate for a variety of products and proposed several research programs the USDA might undertake to clarify the unresolved questions (U.S. Department of Agriculture, 1978a). Acting on the panel's recommendations, the USDA's Food Safety and Quality Service in May 1978 published a final regulation on the use of nitrite in bacon (U.S. Department of Agriculture, 1978b). It specified that sodium nitrite (120 mg/kg) or potassium nitrite (148 mg/kg) be added to bacon along with sodium ascorbate or erythorbate (550 mg/kg) to inhibit nitrosamine formation. It also prohibited the addition of nitrate to bacon. In the same action, it required routine monitoring with a thermal energy analyzer to determine the nitrosamine levels in bacon at its production site. For levels exceeding 10 μ g/kg after cooking, the USDA required confirmation by gas liquid chromatography and mass spectrometry and subsequent monitoring on a lot-by-lot basis until the contamination is reduced to a level lower than 10 μ g/kg.

In 1980, the FDA specified that malt beverages, which had been

halted in mid-1978 by results of an animal feeding study funded by the FDA, which were interpreted as indicating that nitrite per se caused cancer in rats (Newberne, 1978). The existing legislation, i.e., the Food Safety Provisions [Sec. 402(a)(2)(c)] and, for some uses of nitrite, the "Delaney Clause" [Sec. 409(c)(1)(A)] of the Food, Drug, and Cosmetic Act (U.S. Congress, 1980), required that the USDA and FDA proscribe the addition of known carcinogens to foods. Thus, the USDA and FDA made plans for banning nitrite contingent upon further evaluation of the results of the FDA-sponsored study. Subsequently, a group of pathologists established by the Universities Associated for Research and Education in Pathology (UAREP) reviewed the 50,000 histological slides from the animal feeding study and concluded that the initial interpretation was unjustified (Universities Associated for Research and Education in Pathology, 1980). As a result, the USDA and FDA took no action to ban nitrite. However, the potential of nitrite in cured meat products for contributing to the total body burden of nitrosamines remained to be determined.

Thus, in 1980, the USDA and FDA requested that the National Research Council of the National Academy of Sciences examine the current state of knowledge regarding the health effects of nitrate and nitrite in foods and the status of research on alternative curing agents. This report responds to the first part of that request. A second report of the committee will focus on alternative approaches to the current use of nitrate and nitrite.

BOTULISM

A brief review of this disease and its causative organism may be helpful in developing a perspective on the current use of nitrite and nitrate.

Clostridium botulinum is an anaerobic, gram-positive bacterium. The strains of C. botulinum are divided into seven types (A-G), on the basis of their production of antigenically specific neurotoxins (Sugiyama, 1980), and into four groups (I-IV), based on their proteolytic ability and other characteristics (Smith, 1977). Outbreaks of botulism in humans are generally caused by strains of types A, B, E, or occasionally F, in groups I and II. Botulism also occurs in other mammals, birds, and fish (Smith, 1977). The bacterial production of toxin appears to be determined by the presence of a specific bacteriophage, at least in some strains of types C and D C. botulinum (Sugiyama, 1980). Groups I and II have different minimum growth temperatures, heat resistance, and salt tolerance (Genigeorgis and

neurological symptoms such as dizziness, blurred vision, respiratory impairment, and progressive muscular paralysis (Center for Disease Control, 1979; Sakaguchi, 1979).

Food is not the only source of botulism. Toxin synthesis during the multiplication of C. botulinum in a wound or in the gastrointestinal tract of an infant usually less than 6 months of age can also produce the disease (Center for Disease Control, 1979). The symptoms of neurotoxicity in these instances are similar to those of foodborne botulism; gastrointestinal disturbance occurs in infant botulism, but not in wound botulism. The multiplication of C. botulinum accompanied by toxin production in the gastrointestinal tract of adult chickens and rats has been demonstrated by Smart and Roberts (1977), Miyazaki and Sakaguchi (1978), and Sugiyama (1981). Russian investigators have suggested that this process of "toxico-infection" also occurs in humans (Minervin, 1967), but little evidence on this possibility has been collected in the United States. The probability of toxico-infection might be enhanced in humans whose normal gastrointestinal microflora has been disturbed by antibiotic treatment or surgery (Sugiyama, 1981).

The occurrence of the various types of botulism in the United States, their geographic distribution, and their causes have been well documented by the Center for Disease Control (1979). From 1899 to 1977, there were a total of 766 outbreaks of foodborne botulism involving a 1,961 cases, of which 999 were fatal. The average number of outbreaks per year from 1899 to 1949 was 9.7, and from 1950 to 1970 it was 10.3. There were 2.6 cases per outbreak during the first period, and 2.4 during the second period. The proportion of outbreaks in which the toxin type was determined has been increasing over the last few decades. From 1970 to 1977, outbreaks were most often caused by type A toxin (51%), followed by type B toxin (21%) and type E toxin (12%). The toxin type was undetermined in 16% of the outbreaks (Center for Disease Control, 1979).

Since 1950, there has been a gradual decrease in the case-fatality ratio, probably due primarily to improvements in supportive and respiratory intensive care and the prompt administration of antitoxin (Morris and Hatheway, 1980). The case-fatality ratio was significantly higher for individuals 20 years of age or older than for individuals less than 20 (Center for Disease Control, 1979). The ratios were higher for intoxications caused by type E (31%) and type A (24%) than for type B (8.8%) (Feldman et al., 1981).

The information on cases of botulism reported between 1950 and 1979 has been classified into four separate categories. During this period, 215 outbreaks (566 cases) of foodborne botulism occurred,

(60) to commercially processed foods. In 19% of the outbreaks, the type of food processing was unknown (Center for Disease Control, 1979). From 1950 to 1979, the most commonly implicated foods were vegetables (44%), fish and fish products (16%), and condiments (14%) (Feldman et al., 1981). Beef, milk products, pork, poultry, and other vehicles caused fewer outbreaks (Center for Disease Control, 1979).

Tompkin (1980) has reviewed reported outbreaks of botulism attributed to commercially processed or home-processed meat and poultry products in the United States, Canada, and other countries. Since 1899, 15 outbreaks (39 cases, 16 deaths) of botulism in the United States and Canada were attributed to commercially processed meat and poultry products. Seven outbreaks (21 cases, 6 deaths) involved products that are normally cured; the remainder were associated with products that are not normally cured. When information was available on the history of the implicated products, it generally indicated that there had been faulty processing or temperature abuse by the retail outlet or by the consumer (Tompkin, 1980).

Because of similarities to the symptoms of other diseases, botulism may be incorrectly diagnosed as cerebral vascular accident, acute poliomyelitis, myasthenia gravis, chemical poisoning, food poisoning, or acute Guillain-Barré syndrome. Conversely, diseases such as Guillain-Barré syndrome, staphylococcal food poisoning, and carbon monoxide poisoning may be incorrectly diagnosed as botulism (Sakaguchi, 1979).

The dose of toxin causing botulism is too small to stimulate antitoxin production. Thus, it appears unlikely that immunity would develop from repeated low-dose exposures. However, resistance has been observed in certain individuals, such as those with the toxin in their circulatory systems as a result of exposure during outbreaks but with no clinical symptoms. The mechanism of this resistance is unknown (Sakaguchi, 1979).

Factors contributing to the risk of botulism are discussed in Chapters 3 and 10.

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THE UTILITY OF NITRATE AND NITRITE ADDED TO FOODS

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THE UTILITY OF NITRATE AND NITRITE ADDED TO FOODS

Nitrate, nitrite, or both are added with salt (sodium chloride) to a number of foods. This chapter focuses mostly on the utility of nitrite in commercial products of U.S. origin. If nitrate is added, it serves mainly as a reservoir from which nitrite is derived as a result of bacterial reduction. In the United States, nitrite (generally as sodium nitrite) and nitrate are used predominantly in curing red meats and poultry. To a lesser extent, both nitrate and nitrite are used in some species of smoked fish (Chapter 2). Therefore, discussions in this chapter focus on red meats and poultry, and deal only with special considerations pertaining to fish products. The addition of nitrate to some types of cheese -- as practiced in some countries, especially in Europe -- is not permitted in the United States.

Nitrate and nitrite are added to cured products for a variety of purposes. Around 1900, they were shown to be responsible for the "fixation" of color in cured meats (Chapter 2). However, only during the last two or three decades has there been any concerted or extensive attempt to define the specific contributions of nitrate and nitrite to the other sensory and antimicrobial effects of curing.

Many factors complicate evaluations of the utility of nitrite in cured products. For example, there are several reasons for using nitrite to inhibit microbial growth (such as adding nitrite to protect against some foodborne pathogens and to extend shelf life, thereby lengthening the time available for distribution), and their relative importance depends on the assessor's viewpoint. But in practice, the variation in or unpredictability of such factors as time to product consumption and time before the product is subjected to temperature abuse makes it impossible to know the extent to which nitrite must inhibit microbial growth in a product to ensure protection against pathogens or spoilage.

Nitrite is only one of many factors that interact and contribute to a product's sensory properties, such as color, texture, and flavor (i.e., taste and aroma) or its wholesomeness. For example, the composition of a product, including added spices, and smoking contribute to its flavor. Furthermore, the development of spores¹ or growth of

¹Certain microorganisms can exist as spores as well as vegetative cells.

reduces the possibility of contamination by spores or microorganisms and may also affect the proportions of the contaminating microorganisms and their interactions in the developing microflora. Refrigeration may selectively inhibit the growth of microorganisms and reduce the rate of chemical changes, such as lipid oxidation (which is related to rancidity). The characteristics of packaging -- e.g., whether it is oxygen-permeable or impermeable -- may selectively affect the growth of microorganisms or the stability of the color produced by nitrite. Because of these complex interactions, it is difficult to isolate nitrite and assess its contribution to overall product safety or sensory characteristics.

Ideally, experimentation to determine the efficacy of nitrite would include examination of its contribution to the inhibition of pathogenic and spoilage microorganisms, chemical changes, and sensory characteristics in the framework of the variables listed above. Investigators have often focused on a specific action of nitrite in a model system or in a particular product. Caution must be used in extrapolating information from such model systems to commercial products, or from one type of product to another, because differences in conditions, such as water activity and pH, may affect the results.

This chapter briefly describes the current use of nitrate and nitrite in foods and evaluates the scientific evidence of their antimicrobial, antioxidant, and sensory effects. It includes an evaluation of the influence of nitrite and other factors, such as salt and pH, on the germination and outgrowth of Clostridium botulinum spores.

C. botulinum spores are distributed ubiquitously in the environment, e.g., in soil and dust (Smith, 1977). Thus, although it is possible to reduce contamination of carcasses, it is not practicable to produce raw meat with a guarantee that it will never contain spores. Similarly, because of the widespread distribution of C. botulinum in marine and freshwater environments (e.g., Eklund and Poysky, 1970; see also the monograph by Smith, 1977), the committee believes that it is unrealistic to assume that contamination of fish products with C. botulinum can be avoided. Surveys reviewed by Sakaguchi (1979) indicate that fish

²The water activity, a_w , of a food is defined as the ratio of the vapor pressure of water in the food (p) to that of pure water (p_o) at the same temperature, i.e., $a_w = p/p_o$. The growth of some organisms can be inhibited by lowering the water activity of a product (see p. 3-27).

is followed by cell multiplication and may lead to the production of botulinum toxin in foods. If a food containing toxin is ingested without having been thoroughly cooked at temperatures of 80°C or higher, it may cause botulism. Chapters 2 and 10 contain additional discussion of botulism and the events in the sequence that may lead to this disease.

Economic considerations may influence decisions that have an impact on the safety and acceptability of certain products; however, the committee was not asked to assess the possible economic benefits to consumers or producers from the use of nitrite or nitrate nor was it asked to relate scientific evaluation of flavor or color to consumer demand for cured products. The necessity for the current levels of nitrite used to achieve the desired effects in various products will be discussed more fully in the second report of the committee.

THE USE OF NITRATE AND NITRITE

It is difficult to describe the current use of nitrate and nitrite in cured red meats and poultry in the United States because processing, packing, and distribution techniques are constantly changing (Cervený, 1980). Moreover, diverse procedures are used to produce and distribute the wide range of products to which nitrate and nitrite have been added. The size of meat-processing facilities and the sophistication of their quality-control procedures also vary. Many common industrial practices are not described in textbooks nor are they examined in the open literature.

Given these circumstances, any attempt at concise description of the current use of nitrite and nitrate runs the risk of oversimplifying a complex, varied, and ever-changing situation. It is especially difficult now, when new procedures are being sought to reduce residual concentrations of nitrite and, thus, the possibility of food contamination by nitrosamines resulting from the reaction of nitrite and nitrosatable substrates.

It is also difficult to generalize about product classes. Although one can define general categories into which most products will fit, some products may have certain characteristics, such as intermediate water activity, that render them difficult to categorize. The final characteristics of products (such as water activity and pH) may result from traditional manufacturing practices rather than from procedures deliberately designed to produce specific target values for these characteristics. The adoption of "least-cost" formulations may mean that the composition of a product can vary, even over rela-

The following description of the use of nitrite and nitrate in U.S. cured meats and poultry should therefore be regarded as general; in practice, there is much variation in the way the compounds are used. The reader is cautioned that processing procedures for some types of product also vary among countries. For example, English (Wiltshire) bacon, Canadian bacon, and U.S. bacon are taken from different cuts of meat and are processed differently.

Categories of Meat Products

Meat products can be divided into categories based on the extent to which they are heated (if at all) during production, on whether they are cured, and on their water activity. They may also be subdivided into comminuted and noncomminuted (primal) products. The general categories of meat products are briefly described below to facilitate discussion of the role of nitrite in the product and of product susceptibility to effects of microorganisms, which can cause spoilage of the product or make it hazardous to health. The term "processing" is used to indicate manipulation, such as curing or comminution. "Thermal processing" encompasses heating processes that control C. botulinum, but excludes milder heating, such as pasteurization and smoking. "Perishable" products are those that require refrigeration. They may be either raw or pasteurized.

Raw, Uncured Products. This category includes whole or comminuted fresh meats distributed in the uncured state -- products that are not examined in this report.

Raw, Cured Products with High Water Activity (i.e., > 0.92). This category includes raw corned beef packaged with free pickling solution (Price and Schweigert, 1971, p. 465). Other raw, cured products, such as bacon, are subjected to some smoking and mild heating during production (Kramlich et al., 1973, p. 228).

Raw, Cured Products with Low Water Activity (< 0.92). Scotch, prosciutto, Westphalian, and country hams; dry-cured bacon; and dried sausages may be cold-smoked and not heated appreciably during processing. Other products in this category, such as some sausages, may be mildly heated if smoked (Kramlich et al., 1973, p. 228; Nitrite Safety Council, 1980). These products are sold as raw, cured products with a low water activity due to drying and the addition of salt. Many dried meat products such as dried beef, jerky, dry-cured bacon, dry-cured ham, and many dried sausages are produced with added nitrite. Many of these products depend on relatively

Cooked, Uncured Products. This category includes some meat loaves, some loaves containing meat and other ingredients, poultry rolls, bratwurst, and ring liver sausage (Kramlich et al., 1973, p. 98). During processing, most of these products are subjected to temperatures of 65°C or higher in order to pasteurize them. They are not cooked sufficiently to destroy C. botulinum spores.

Cooked, Cured Products. This is by far the largest category of products processed with added nitrite (Price and Schweigert, 1971, p. 485). Pasteurized products, heated to 65-75°C center temperature, include hams in casings and in cans, frankfurters, bologna, liver sausage, meat loaves, some loaves containing meat and other ingredient and some roll products. Bacon is classified as a raw, cured product because it is generally not heated sufficiently for pasteurization, but its microbial profile more closely resembles that of cooked, cured products. A second class of cooked, cured products includes the so-called "shelf-stable" products, such as canned meats and prefried canned bacon (Cervený, 1980). These products are normally heated to a center temperature of 95-112°C. This alone is not sufficient to kill all spores of C. botulinum or other organisms, but in conjunction with other factors, such as the presence of nitrite, heating to this extent can delay spore outgrowth. Many of these products contain not only meat but also many other ingredients. Other products in this class are luncheon meat, hams, and pork shoulders.

There are only a few "commercially sterile" cured products. Among these are corned beef hash, deviled ham, meat spreads, and Vienna sausages. These "commercially sterile" products are defined as products free of pathogens as well as of microorganisms capable of growing under normal nonrefrigerated storage conditions. They are thermally processed to the extent that the slowest heating portion of the can receives a treatment that is at least as destructive to the most resistant C. botulinum spore as treatment at 2.78 min at 121°C (page 3-22).

Annual Production of Cured Meat Products

The major cured meat products to which nitrite is added and the general methods of addition are shown in Table 3-1. As the table illustrates, nearly 4 billion kilograms of these products were processed with added nitrite in the United States during 1979. Table 3-2 lists the amounts of meat products processed without added nitrite. Although the use of nitrate in products has been declining (Binkerd and Kolari, 1975; Cer en . 1980; Sofos and Busta, 1980), it

Meat Products Processed with Added Nitrite Under Federal
Inspection in the United States During 1979^a

	<u>U.S. Production, billions of kilograms</u>	<u>Sodium or Potassium Nitrite Added,^b mg/kg</u>	<u>Most Probable Method of Adding Nitrite</u>
beef	0.13	200	Multineedle injection
at canned	0.83	200 ^c	Multineedle injection
	0.76	120	Multineedle injection
e:			
dry and dry	0.15	156	Direct addition as cure
kfurters	0.68	156	Direct addition as cure
gna	0.37	156	Direct addition as cure
es, cured			
at	0.05	156	Direct addition as cure
es, mixed			
at and			
nmeat			
gredients	0.09	156	Direct addition as cure
r	0.06	156	Direct addition as cure
r cooked			
ems	0.38	156	Direct addition as cure
Meats:			
	0.13	200	Multineedle injection
heon meats	0.13	156	Direct addition as cure
a	0.04	156	Direct addition as cure
na sausage	0.05	156	Direct addition as cure
ellaneous	0.05	156	Direct addition as cure

from American Meat Institute, 1980, and U.S. Department of Agriculture,

e numbers are subject to adjustment for the weight of nonmeat ingredients,
at loss during cooking, and production in other than federally inspected
s.

t of nitrite added to meat products is based on amount of meat in the
lation. Thus, as extenders and other nonmeat ingredients are increased,
te added to total product is decreased. Most commonly, sodium nitrite
e nitrite salt used

TABLE 3-2

Meat Products Processed without Added Nitrite Under Federal
Inspection in the United States During 1979^a

<u>Product</u>	<u>U.S. Production, billions of kilograms</u>
Beef, cooked	0.11
Steaks and chops (chopped and formed)	0.13
Meat patties	0.21
Hamburger, ground beef	1.29
Other meat products	0.42
Pizza	0.21
Pies	0.08
Dinners	0.12
Entrees	0.17
Other products containing meat	0.13
 Sausage:	
Fresh beef	0.01
Fresh pork	0.37
Other fresh sausage	0.10
 Canned:	
Chile con carne	0.15
Meat stew	0.07
Pasta meat products	0.20
Other	0.23

^aData from American Meat Institute, 1980, and U.S. Department of Agriculture, 1980. These numbers are subject to adjustment for weight of nonmeat ingredients, weight loss during cooking, and production in other than federally inspected plants.

(brown-and-serve, Canadian-style, country-cured, country-style, jowl, pancetta rolled, pork shoulder); **fatback; hocks; jowls; loin; roll; shoulder butt;** der picnic; hams (canned, chopped, chopped loaf, ess, country-style, cured, prosciutto, roll, semi-ess, shanks, sliced, sliced boneless, Westphalian); d, chopped; spareribs

TRY

ed parts, carcasses, products; sandwiched; smoked; smoked and stuffed; cured turkey ham

AGES

ed; cooked and smoked; dry; for pizza; Italian (smoked); Italian (cooked, cured); liver; New England id; Polish (bratwurst); pork; semidry; smoked; smoked, dry; summer; summer (cooked); summer (dry)

BEEF

Bologna (beef, garlic, Lebanon); breakfast beef; corned beef; corned beef brisket; corned beef creamed, chipped; cured; frankfurters; in br **jerky, meat bar; patties; roll, cooked, corn** smoked; sticks; tenderloin steaks; tongue, c and smoked

SALAMI

Cooked; cotto; dry, hard; Genoa; German (dry) (dry, hard)

OTHER PRODUCTS

Beerwurst; braunschweiger; bratwurst; cannell tortellini; cappicola; cervelat; chorizo, dry fraizzes; galentini; head-cheese; sailcicca cheese; Holsteinen; kielbasa; knockwurst; la linguisa; cured meat loaves (beef, ham and minced, olive, pepper, pepperoni, pickle and pimento); longaniza; meats (e.g., poultry in and soy protein product, casseroles, cured m cured patties, dehydrated, dressing with meat poultry, in a blanket, luncheon, macaroni wi meat and gravy, omelets with meat or poultry components, or poultry, vegetables in gravy, salads, soup with meat, spreads); Milano; mo (cooked); pastels; pastrami; special items (corn dogs, crepes, enchiladas, hors d'oeuvre products, lima beans smoked--pork, ham, baco pate, pizza, quiche product, sauerkraut prod veal cordon bleu)

ducts were identified from labels, approved by the U.S. Department of Agriculture from 1979 to 1981, on create was listed as an ingredient. Data from U.S. Department of Agriculture, 1981, personal communication

regions of those countries is nearly identical. However, the use of nitrate in Canadian meat products has been forbidden, with some exceptions, for several years (Health and Welfare Canada, 1975).

Use of Nitrite in Fish Products

Total production of commercially smoked fish products in the United States in 1979 was 9,564,900 kg (National Oceanic and Atmospheric Administration, 1980). Products in which nitrite was allowed constituted 5,750,000 kg of this total. Thus, in comparison with the volume of cured red meats and poultry, the amount of fish in which nitrite was used was relatively small. On the basis of a total population of 218 million, the 1979 consumption of smoked fish products was 43.9 g per person. For products in which nitrite is allowed, the per capita consumption was no more than 26 g. Despite the relative productions of smoked fish and cured meat products in the United States, there have been more recent outbreaks of botulism attributed to the former (Center for Disease Control, 1979).

Use of Nitrate in Dairy Products

Cheese of the Gouda and Edam types, as produced in some European countries, are very susceptible to late blowing (swelling) as a result of the proliferation of clostridia (Galesloot, 1961, 1964; Gray et al., 1979). One of the most successful methods of preventing late blowing of cheese is the addition of potassium or sodium nitrate (Galesloot et al., 1975). The addition of nitrate has been shown to inhibit the development of clostridia shortly after the cheese is immersed in brine when the water activity of the cheese is high. During this period, the low salt content permits the most active spores to germinate. The germinating spores are very susceptible to nitrite, which is produced from the added nitrate primarily by action of the milk enzyme, xanthine oxidase (Galesloot, 1961). So few spores remain that their germination can be controlled by the salt concentration, which gradually increases as the brine penetrates the cheese.

Dutch cheesemakers are permitted to add 15 g of sodium nitrate per 100 liters of milk. In Canada, a recent change in regulations permits the addition of 20 g of sodium nitrate per 100 liters of milk in the manufacture of some cheeses. The nitrate contents of different varieties of cheeses are well documented in the literature (Brathen and Svensen, 1973; McKay, 1974; Rammel and Joerin, 1972); however, there have been few reports related to the course of nitrate degradation and formation of nitrite during ripening of these cheeses.

after 6 weeks. After this period, only a slight further decrease was observed. The nitrite ion content of these cheeses was very low -- a maximum of approximately 1 mg/kg after 2 to 3 weeks. Even cheese prepared from milk containing sodium nitrate at 60 g sodium nitrate per 100 liters contained nitrite ion at only 1.5 mg/kg when analyzed 14 weeks after manufacture.

The practice of adding nitrate to cheese milk has been criticized for constituting a health hazard on the grounds that it may lead to the formation of nitrosamines. Most data indicate that the concentrations of nitrosamines in cheeses made with added nitrate range from 1 to 5 $\mu\text{g/kg}$. However, nitrosamines are also found in cheeses without added nitrate (Gray et al., 1979). In only a few studies have the concentrations exceeded 10 $\mu\text{g/kg}$. In those cases, the methods of analysis were not specific, nor were they sensitive enough for the purpose (Cantafora et al., 1974; Cerutti et al., 1975).

The potential that exists in Europe for clostridial spoilage of cheese does not appear to constitute a major problem for products made in the United States, where the addition of nitrate (or nitrite) to cheese is not permitted. However, the nitrate added to imported cheese is relevant to the intake of nitrite and nitrate by the U.S. population (Gray et al., 1979). This is discussed in Chapter 5.

Methods of Adding Nitrite to Meat Products

Nitrite is added to meat products either as a nitrite salt, usually sodium nitrite, in a nitrite-containing curing salt mixture, or in a solution of nitrite and other ingredients, which is referred to as "pickle." The method of addition may affect the uniformity of the distribution of nitrite and, thus, the minimal concentration needed to achieve consistent results, e.g., in color development.

For most intact or "primal" (chunk) products that are made from portions of meat that weigh from 100 to 200 g or more, the pickling solution containing nitrite is injected into the product. Multineedle injectors are most commonly used for boneless products and sometimes for bone-in products (Kramlich et al., 1973, p. 58). In general, according to good manufacturing practices, the pickling solution is made up daily (Komarik et al., 1974, p. 2). It typically contains water, salt, sugar, phosphate, ascorbate (or isoascorbate), and nitrite (Kramlich et al., 1973, p. 40) and is produced by using a sodium chloride carrier that contains approximately 6.25% sodium nitrite or by adding sodium nitrite to the solution to be used for the pickle. It is often advantageous for the processor to use sodium nitrite, if

pickle in the processed product. Some boneless hams and cured beef products are subjected to mechanical treatment -- tumbling or massaging -- after injection of the pickling solution in order to distribute the solution more uniformly and to enhance protein functionality in the processed product, thereby promoting product uniformity and tenderness and facilitating slicing and other portioning of the product.

For comminuted products, the nitrite or nitrite-containing curing salt is generally added directly during blending. Often, the meat is ground and blended with salt, nitrite, or curing salt and water. This process results in a very uniform distribution of the nitrite. During the manufacture of sausage, the meat is ground again and blended or chopped to the desired consistency. The coarsely comminuted products are used for dry and semidry sausages and for some loaf products. Very finely comminuted emulsions are used for a variety of skinless frankfurters, bologna, and loaves.

Nitrite or nitrate (or both) may be applied to some products as part of a dry rub (Kramlich et al., 1973, p. 52; Price and Schweigert, 1971, p. 463). This rub usually contains salt, sugar, and nitrite or nitrate (or both), which are blended and then mechanically or manually applied to hams, pork bellies, or beef. Much time is needed for the diffusion of the cure throughout the product and its subsequent drying. In the United States, at least 45 million kilograms of such products are produced each year. Dry-cured products are often traditionally consumed by populations in specific geographic areas, where they are usually manufactured by small- and medium-sized processors.

The Fate of Nitrite in Meat

Typical fresh muscle consists of approximately 70% water, 20% protein, 9% fat, and 1% analyzable ash. The composition may vary widely, depending on the particular muscle or cut of meat, the species, the nutritional state of the animal, and other factors.

From a structural viewpoint, meat consists primarily of myofiber, but it also contains fat cells, fibroblasts, endothelial cells, and neural cells. All cells are held in place by extensive connective tissue. In living muscle, the components are compartmentalized by the structure, but postmortem conversions may allow more freedom (not necessarily unrestricted) for the movement of chemicals. The presence of fat cells in meat entails lipid-polar interfaces that may facilitate certain chemical reactions (Cassens et al., 1979b).

bookkeeping or balance experiments have been attempted to determine at least generally, how much of the nitrite remains in the meat and how it is distributed. As long ago as 1940, there was interest in the decomposition or loss of nitrite, especially as a result of heating. Greenwood (1940) suggested that the aliphatic diazo reaction could be important in the loss upon heating.

Nitrogen has been selected as the marker in such experiments. Two problems have impeded progress. First, radioisotopes of nitrogen have such a short half-life that they cannot be used, so the stable nitrogen-15 has been used. Second, meat is an extremely complex system and, because many kinds of techniques are used for processing cured meat, simple all-encompassing answers have been difficult to obtain.

Two groups of investigators have expended considerable effort using [^{15}N]-nitrite in attempts to learn about its fate. Japanese workers have published a series of papers on the topic. Using a model system of myoglobin, nitrite, and ascorbate, Fujimaki et al. (1975) recovered all [^{15}N]-labeled nitrite-nitrogen added to the system in the forms of residual nitrite, nitrate, the nitrosyl group of denatured nitrosomyoglobin, and gaseous nitrogen. However, when they used a meat system, recovery fell to a range of 66% to 90% (Emi-Miwa et al., 1976). When sodium ascorbate was added to the system, recovery was even lower. The authors pointed out that much of the nitrogen-15 was found in the water-soluble and salt-soluble protein fractions and that a substantial amount was converted to gaseous forms.

Sebranek et al. (1973) reported results of experiments in which they traced nitrogen-15 from $\text{Na}^{15}\text{NO}_2$ added to commercially cured products. They concluded that the added nitrite was changed rapidly to other compounds when it was added to meat and that little of it escaped from the product in volatile form. After processing, the change continued, but at a slow rate, until the concentration of residual nitrite was low. Subsequent studies were conducted by the same group to determine the percent of added label (from $\text{Na}^{15}\text{NO}_2$) that can be recovered in a given portion of meat shortly after processing (Goutefongea et al., 1977; Woolford and Cassens, 1977; Woolford et al., 1976). The results were: with myoglobin, 5-15%; as nitrite, 5-20%; as nitrate, 1-10%; as gas, 1-5%; with sulfhydryl groups, 5-15%; with lipid, 1-5%; and with non-heme protein, 20-30% (Cassens et al., 1977). It is not known if these percentages change as the time after processing increases. Cassens et al. (1979a) have recently reviewed what is known about the reactions of nitrite in meat and some of the issues yet to be clarified.

its solubility. Walters et al. (1979) reported that nitric oxide can react with unsaturated fatty acids. They suggested that pseudonitrosamine is formed across the two double bonds in palmitodiolein. When the latter compound was heated with morpholine in lipid solvent, nitrosation of the secondary amine occurred. The authors suggested that a similar mechanism may lead to the formation of N-nitrosopyrrolidine in bacon.

When nitrite is added to meat immediately after processing, only about 50% is detectable as residual nitrite by the usual analytic methods. The chemical forms of this residual nitrite detected by those methods are not known, nor are the forms and reactivity of the added nitrite that is not detectable as residual, including the portion of the added nitrite that could not be accounted for in some balance studies. These issues are discussed in Chapter 5. The measured residual nitrite undergoes depletion as the product is stored. The depletion is faster at abuse temperatures -- e.g., room temperature -- than when refrigerated (Nordin, 1969) and in the presence of reductants (Fox and Nicholas, 1974). The importance of added and residual nitrite in the inhibition of C. botulinum is discussed below.

Postprocessing Handling of Cured Products: Factors Influencing the Requirement for Preservatives

Cured meat products are often distributed through complex distribution chains. A product from a national packer may have to pass through the packer's warehouse, a broker, a retail-chain warehouse, and a retail store. Cooked, cured, vacuum-packed, and refrigerated meat products must have a 30- to 60-day shelf life. In Europe, the distribution chain may be located in a relatively small geographic area, so the shelf life may not need to be that long. This may also apply in the United States, if local and regional processors are preparing products that will be consumed within 2 weeks.

Foods are frozen and refrigerated more extensively in the United States than elsewhere. In this country, most raw, uncured meats or meat products are distributed or stored either refrigerated or frozen and many raw or pasteurized cured products are also refrigerated. Meats that are cured with nitrite and salt are generally not frozen because freezing longer than several months would result in the development of a rancid flavor (Kramlich et al., 1973). However, some cured meats may be stored frozen, e.g., by the Armed Forces, for limited periods. Consumers may buy large quantities of cured meat products and keep them in home freezers for long periods. Freezing increases the shelf life of a product.

on the loading dock), in transit during distribution, in a warehouse, in a retail store (e.g., at the top of a display case), or in the possession of the consumers (e.g., in a car trunk or in an insufficiently cold refrigerator).

The time between production and consumption of nitrite-cured products may vary tremendously. Some pasteurized canned hams might not be consumed earlier than a year after manufacture. During this time, the residual nitrite will decline considerably, possibly to levels too low to be detected. Some products with added nitrite may be consumed shortly after they are removed from the heat-processing equipment in the manufacturer's plant. Thus, the time to consumption of cured products, which is not subject to regulation, may vary from less than 1 day to more than 1 year after production. Because of this great variation in the time to consumption and the unpredictability of the timing and duration of abuse, it is not possible to specify the degree to which a preservative should inhibit microbial growth to ensure product safety.

MICROBIOLOGICAL EFFECTS OF NITRITE

Preservation

The method used to preserve a meat product frequently determines the potential for microbially induced spoilage or hazard to human health (International Commission on Microbiological Specifications for Foods, 1980, pp. 333-409). Raw meats stored under chilled conditions exhibit spoilage patterns that are fundamentally different from those of frozen or dried meat products. Raw cured meat products support growth of various microorganisms, but the water activity largely determines the flora that develops. In heated products, the potential for microbial proliferation is determined by a complex interaction involving the degree of heating, the presence of curing salts, and the characteristics of the product.

Meat Spoilage. Table 3-4 lists some of the effects of microbial activity observed in red meat products in temperate climates. These spoilage patterns illustrate that the preservation method used determines the type of microbial spoilage that occurs.

Sources of Contamination. Meat products may be contaminated any time from the moment of slaughter through all processing and handling procedures. Equipment, personnel, additives, and other environmental contacts serve as possible reservoirs of contamination. There are also numerous opportunities for contamination by microorganisms from the intestinal tract, the lymphatic system, and the outer surfaces

<u>Product Category</u>	<u>Description of Defect</u>	<u>Microorganism</u>
<u>Fresh Meat</u>		
Fresh, refrigerated (0°-5°C)	Off-odor, slime, discoloration	<u>Pseudomonas</u> , <u>Aeromonas</u> <u>Alcaligenes</u> , <u>Acinetobacter</u> , <u>Microbacterium</u> , <u>Moraxella</u> , <u>Proteus</u> , <u>Flavobacterium</u> , <u>Alteromonas</u> , <u>Saccharomyces</u>
	Lipolysis, pungent odor	<u>Pseudomonas</u> , yeasts
	Moldy	<u>Penicillium</u>
	Whiskery	<u>Thamnidium</u>
	Black spot	<u>Cladosporium</u>
	White spot	<u>Sporotrichum</u>
Fresh (15°-40°C)	Bone taint	<u>Clostridium</u>
	Gassy	<u>C. perfringens</u>
	Foul odor	<u>C. bifermentans</u> , <u>C. histolyticum</u> , <u>C. sporogenes</u>
Vacuum packed	Acid, sweet, rancid	<u>Lactobacillus</u> , <u>Microbacterium</u> , <u>Enterobacter</u> , <u>Hafnia</u>
<u>Cured Meat:</u>		
Bacon	Cheesy, sour, rancid	<u>Micrococcus</u>
	Discoloration	Molds
	Slight souring	<u>Lactobacillus</u> , <u>Micrococcus</u> , <u>Vibrio</u> , <u>Alcaligenes</u> , <u>Corynebacterium</u>
	Putrefaction	<u>Clostridium sporogenes</u>
Vacuum packed	Cabbage odor	<u>Proteus inconstans</u>
	Tainted	<u>Vibrio</u>
Brines	Turbid	<u>Debaryomyces</u> , <u>Kloeckera</u>
Ham	Surface slime	<u>Micrococcus</u> , <u>Microbacterium</u> , Yeasts
	Gassy or puffy	<u>Clostridium</u>
	Green discoloration	<u>Lactobacillus</u> , <u>Streptococcus</u> , <u>Leuconostoc</u>
	Bone and meat "sours"	<u>Clostridium</u>
	Surface	Molds

(Table continued on next page)

TABLE 3-4 Continued

<u>Product Category</u>	<u>Description of Defect</u>	<u>Microorganism</u>
<u>Cured Meat (Cont.):</u>		
Sausages	Slime on surface	<u>Micrococcus</u> , yeasts
	Gas production (vacuum packed)	<u>Lactobacillus</u>
	Greenish discoloration	<u>Lactobacillus</u> <u>viride</u>
		<u>Leuconostoc</u>
Fermented sausages	Slime	Yeasts
	Spots	Molds
<u>Canned Meat:</u>		
Commercially sterile	Gas, putrefaction	Spore-formers (e.g., <u>Bacillus</u> , <u>Clostrid</u>
Semipreserved	Souring, discoloration	<u>Streptococcus</u>
	Putrefaction, gas	<u>Bacillus</u> , <u>Clostridium</u>

^aAdapted from Banwart, 1979, pp. 430-431.

intoxications as well as disease-producing microorganisms for which the meat serves solely as a vector. After processing, distribution to retail outlets, and subsequent sale to the consumer, microbial contamination may occur during storage, food preparation, and handling immediately before consumption. The source(s) of contamination frequently determine the range of microbial contaminants present and, thus, the potential spoilage or hazard.

Potential Health Hazards. Pathogenic microorganisms or the toxins they produce, or both, are potential hazards associated with meat products. The frequency with which meat products are implicated in foodborne illnesses has been reported in annual summaries produced by the Centers for Disease Control (1981). Bryan (1980) has also summarized and analyzed surveillance data on reported outbreaks of

Some products are subjected to heating, e.g., pasteurization, which does not kill all pathogens. Subsequent inappropriate storage or handling of these products could result in foodborne illnesses. Recontamination, e.g., by salmonellae, coupled with mishandling can also lead to a foodborne infection, e.g., salmonellosis (Bryan, 1980).

Certain conditions can favor the survival and growth of anaerobic spore-formers. These conditions include the elimination of competition by the killing of vegetative cells, especially those of non-spore-formers, which can occur when products are thermally processed. In these circumstances, the most serious potential hazard might arise from C. botulinum, the toxin from which may cause botulism. However, C. perfringens, Bacillus cereus, and other spore-formers may also pose a hazard. In some situations, when competition from other organisms is minimized, contamination by Staphylococcus aureus followed by mishandling could permit the production of a heat-resistant enterotoxin that could result in a foodborne intoxication. Even if products are resistant to most bacterial invasions, mycotoxins could be produced during mold growth, and, consequently, mycotoxicosis could be a threat to the consumer. One could speculate that gastroenteritis might be caused by Campylobacter fetus subsp. jejuni, Yersinia enterocolitica, enteropathogenic Escherichia coli, and similar pathogens if these organisms contaminate meat and if the conditions are favorable for infection of the consumer. Red meats have not yet been implicated as a cause of such illness; however, poultry may be responsible for some outbreaks of illnesses caused by C. fetus (Bryan, 1979, p. 265.) Curing procedures that effectively control some of these hazardous microorganisms are discussed below and are listed in summary Table 3-7 at the end of this chapter.

The Effect of Microbial Growth Patterns on Spoilage or Hazard

Microbial growth patterns must be understood in order to evaluate food preservation methods and to assess potential health hazards. Most microorganisms in meats multiply by binary fission, i.e., they propagate by dividing into two daughter cells. Some microorganisms, such as yeasts and molds, multiply by budding daughter cells or by forming fruiting bodies with multiple propagules; however, these differences do not significantly modify the pattern of overall population growth.

Characteristic growth patterns are shown in Figure 3-1. An

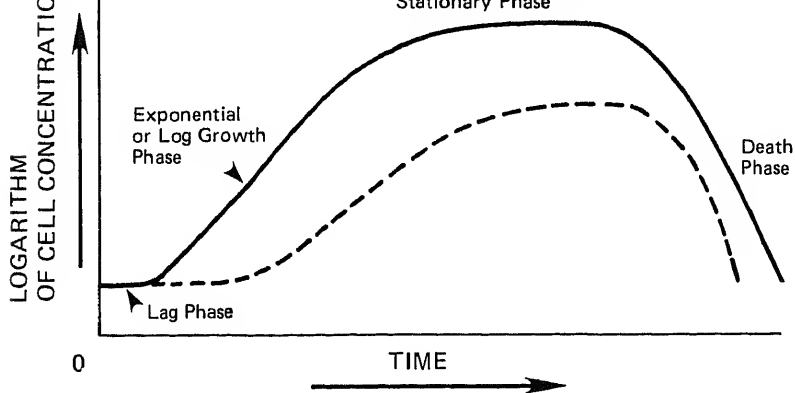


FIGURE 3-1. Microbial growth patterns. Solid line represents the hypothetical growth under ideal conditions. The broken line represents the hypothetical growth under unfavorable conditions.

or logarithmic phase. The growth rate and length of the lag phase are dependent upon many factors. When conditions are not optimal for growth (e.g., reduced or elevated temperatures, dehydration, inadequate nutrients, and the presence of chemical inhibitors of growth such as antibiotics, nitrite, etc.), the lag phase may be extended for lengths of time corresponding to the severity of the antagonistic environment. If growth commences under these unfavorable conditions, the growth rate during the exponential phase may be slower or the population increase smaller. Antagonistic conditions may also increase death rates in the final phase. These differences in growth patterns are illustrated in Figure 3-1.

The initial concentration of contaminants influences the length of time needed for the development of detectable or undesirably large populations. Large populations of specific microorganisms are required to induce spoilage of products. Infection of a host requires a minimum population of pathogenic microorganisms that can establish and colonize a specific organ or tissue. The size of this minimum population varies among pathogens. Response to a microbially produced toxin is dose dependent, and the concentration of the toxin is often directly related to the number of cells capable of producing it. Sometimes, these toxins are not produced until late in the growth phase of a microbial population. Successful competition of a specific microorganism depends upon its ability to outgrow the competitive

undetectable; they can range from a few minutes to several days. During the exponential phases of growth, populations of some species can double in 8 minutes under ideal conditions or the process may take weeks under suboptimal conditions. Initial population levels can be as low as one cell per kilogram of product or as high as levels approaching those observed in the stationary phase, which frequently range from 10^6 to 10^{10} per gram of growth substrate.

In meat products, unless some effective or severe processing procedure selects for one kind of microorganism, a mixed flora usually predominates. This means that several species and strains of microorganisms may be competing for domination of the meat product. However, "starter" cultures are added to some products to establish a desired flora rapidly.

It is possible to have one or more of a variety of cultural interactions ranging from symbiosis to neutrality to synnecrosis. Consequently, there may be spoilage microorganisms that inhibit growth of pathogens, there may be lactic acid starter cultures that are effective protective agents, or there may be no competition permitting pathogens to proliferate.

Factors Affecting Growth. Many factors influence the growth of microorganisms, and various species of microorganisms have dramatically different nutritional requirements. The composition of the food or meat product determines the substrates available for growth. Temperature is critical in the regulation of growth rates and survival. Physical factors, such as the water activity (a_w), the pH, the oxidation-reduction (redox) potential (E_h), and the gaseous environment also have a substantial influence on growth. Closely linked to these factors are the species of solutes (e.g., acids, curing adjuncts, salts, and sugars) and the antimicrobial agents (both added preservatives and naturally occurring inhibitors) that are present in the microenvironment immediately surrounding the cells. Finally, the effects of processing procedures and the interactions of these many factors must be considered when examining the growth of microorganisms.

Contributions to the Control of *C. botulinum* and Other Clostridia

Many moderate heat-processing treatments or other factors that stress cells will inactivate most vegetative bacterial cells and eliminate competition, but allow viable spores, including those of *C. botulinum*, to remain in the meat system. Frequently, these treatments will also activate spores for subsequent initiation of germination, which, under adequate conditions, will lead to outgrowth, cell division, and ultimately toxin production in the case of *C. botulinum*.

rather specific requirements for growth and toxin production. In a mixed microflora, it is moderately competitive, but less so as pH is lowered. Consequently, it benefits from processes that eliminate competition and reduce the E_h of the system favoring anaerobic growth. Cured meats, especially heat-processed cured meats, provide a near optimal balance of nutrients, growth environment, reduced competition, and many of the other factors that promote growth of C. botulinum (Sakaguchi, 1979).

A variety of physical and chemical factors determine whether C. botulinum proliferates and forms toxin in cured meats. Many of these factors may also control the proliferation of spoilage organisms or other pathogens. They include thermal processing; level of C. botulinum spores and vegetative cells at time of abuse; concentration of sodium chloride; pH of the meat; concentration of carbohydrate; storage temperature; water activity or brine level

$$(\% \text{ brine} = \frac{\% \text{ salt}}{\% \text{ salt} + \% \text{ moisture}} \times 100, \text{ also defined in some reports as } \% \text{ salt per unit volume});$$
 presence and concentration of ascorbate and isoascorbate (erythorbate), including their effect on the level of available iron in the product; input and/or residual level of nitrite; other curing adjuncts; and packaging. Certain of these factors interact in exerting control over microorganisms. Such interactions are described later in this chapter.

Thermal Processing. Spores of pathogens such as C. botulinum and putrefactive spoilage organisms can be inactivated by thermal processing. Spores of some putrefactive, but nonpathogenic microorganisms commonly found in foods are more heat-resistant than those of C. botulinum (International Commission on Microbial Specifications for Foods, 1980, pp. 1-37, 136-159). Thus, the latter will be controlled if the former are.

Thermal processing is used extensively in the production of a variety of food products. A "botulinum cook" is a 12D process,³ i.e., one that will inactivate 99.999999999% of the C. botulinum spores in any volume. To assign an appropriate thermal process for specific foods, a number of assumptions must be made based on empirical findings. Among these are the assumptions that the spores have a uniform level of heat resistance, that they are uniformly distributed throughout the product, and that inactivation proceeds with linear, first-order kinetics. If one were to assume a normal population of 10^2 spores per can, a 12D process would leave one surviving spore in a total of 10^{10} cans of products.

To ensure that thermal processing is adequate, the temperature and duration of heating are measured and safety margins incorporated into the treatment. However, the reliability of thermal processing is compromised most frequently by failure to deliver the intended treatment rather than by faulty process design. The cause of the failure to deliver the intended treatment can be mechanical, e.g., defective materials and equipment, or human error, e.g., mismeasurement of temperatures. The Hazard Analysis and Critical Control Point Program currently recommended by the regulatory agencies has contributed greatly to increasing the reliability of thermal processing (Genigeorgis and Riemann, 1979).

To obtain a 12D inactivation of C. botulinum spores in phosphate buffer at pH 7.0, the thermal treatment must be administered for 2.78 minutes at 121°C. For C. botulinum, it is generally accepted that a decrease or increase of 10°C from this temperature will produce a tenfold decrease or increase in the number of spores killed (Genigeorgis and Riemann, 1979). Accordingly, 10 minutes at 111°C or 0.1 minute at 131°C are both equivalent to 1 minute at 121°C. By custom, a heat treatment of 1 minute at 121°C has been accorded a "lethal value" (F_0) of 1 (Hauschild, 1980).

Thus, the "botulinum cook" is designated as having a lethal value (F_0) of 2.78. If one knows the length and temperature of treatment and that lethality varies logarithmically with change in temperature, the lethal value of any thermal process can be calculated. For low acid foods, e.g., canned mushrooms, thermal processes of $F_0 > 2.78$ (or, often, double that value) are used to prevent spoilage losses from organisms whose spores are more resistant to heat than those of C. botulinum (International Commission on Microbial Specifications for Foods, 1980, pp. 1-37).

The sensory characteristics (e.g., color, flavor, and texture) and functionality of most cured meat products would be unacceptable after thermal processing to $F_0 = 2.78$ or even after a lower level of processing to $F_0 = 1.0$. Thermal processes with F_0 values ranging from 0.05 to 0.4 are frequently used for shelf-stable cured meat products. These lethal values are from 10 to 100 times lower than those apparently required for a minimal "botulinum cook" for low acid foods. Consequently, the process must be highly dependent upon other inhibitors such as nitrite and sodium chloride to ensure safety and freedom from spoilage (Lechowich et al., 1978; Pivnick et al., 1969).

Thermal processing of most cured meat products has been successful because of the supplementary effects of nitrite, salt, and the very

Current thermal processing practices used by industry to control pathogens and spoilage reflect earlier comprehensive studies by Stumbo et al. (1945a,b,c), Gross et al. (1946a,b), Vinton et al. (1947a,b), Schack et al. (1959), and Pivnick et al. (1969, 1970).

In reviews of effective thermal processes for controlling bacterial spores in canned cured meats, Duncan (1970) and Riemann (1963) have reaffirmed earlier observations that the stability of cured meats is dependent upon a complex interaction between the heat treatment and the curing agents. Pivnick et al. (1969) described the interaction of salt, nitrite, the number of C. botulinum spores in the inoculum, and severity of heat treatment on subsequent toxin production. A thermal process with an F_0 of 0.15 did not control subsequent toxin production when there were 10^4 spores per gram of ground cured pork. A treatment of $F_0 = 0.3$ was adequate for 10^4 spores per gram, but was insufficient for 10^6 spores per gram, which could be controlled by an F_0 of 0.6. Viable botulinum spores were recovered after 18 months, even in a product that was unspoiled and nontoxic. Meat devoid of curing salts (i.e., sodium chloride and sodium nitrite) was inoculated with one spore per gram and processed to an F_0 of 0.62. Upon incubation it became toxic.

Heat-damaged spores of clostridia or Bacillus spp. are more sensitive to the effects of curing salts than are undamaged spores (Roberts and Ingram, 1966; Roberts et al., 1966). Spores of C. botulinum subjected to a sublethal heat process at 95°C were inhibited by unheated nitrite less than by a nitrite-containing medium that had been heated at 115°C for 15 minutes (Ingram and Roberts, 1971). These responses were not observed in a model meat system heated at lower temperatures (80°C) for longer times (4 hours) (Ashworth et al., 1973). Jarvis et al. (1976) suggested that although spores heated and damaged at higher temperatures of 90°C might be sensitized to nitrite and salt, those subjected to the lower temperatures used for pasteurization (63°C to 74°C) might not be sensitized. Furthermore, inhibitory effects produced upon heating were much more evident in some laboratory media than in meat systems (Johnston and Loynes, 1971; Perigo et al., 1967). Thus, precaution should be used when extrapolating laboratory data to commercial meat processing.

Thermal processing is generally regarded as encompassing only those heating procedures which have a preservative action against C. botulinum. Pasteurization processes with temperatures ranging from 63°C to 74°C have no direct effect on spores of C. botulinum in cured, perishable comminuted meats (Tompkin et al., 1978b); however, such temperatures will kill many types of germinated spores or

contaminated will affect the likelihood of its spoilage and the adequacy of the protection provided by nitrite and other factors.

Skovgaard (1980) and Holley (1978, 1981) reviewed various reports indicating that meats may occasionally contain low levels of C. botulinum. In the United Kingdom, investigators studied a series of samples from one processor and reported that an average of 1 to 2 C. botulinum cells were present per kilogram of bacon and that sporadic contamination much higher than this average also occurred (Roberts and Ingram, 1977; Roberts and Smart, 1976a, b). However, the authors of a survey of random samples of commercial bacon in Canada suggested that the most probable number of C. botulinum cells was 0.064 per kilogram (Hauschild and Hilsheimer, 1980). The apparently low incidence of C. botulinum cells may be partially responsible for the excellent public health record associated with the consumption of cured products; however, research on this subject has not been systematic.

It has been well documented that typical heat processing and curing agents used for perishable or shelf-stable cured meats may be inadequate when the meat is heavily contaminated with C. botulinum spores (Christiansen et al., 1973; Pivnick et al., 1969; Sofos et al., 1979a).

Sodium Chloride. The final concentration of sodium chloride in most cured meat products is approximately 2 to 3% of the total product weight, which corresponds to approximately 3 to 6% salt in the aqueous phase (i.e., a 3 to 6% brine concentration) for most products. The brine concentration will, however, vary with the moisture content of the product. In meat products, it is the concentration of salt in the aqueous phase, rather than the percentage of salt based on the total product weight, that is the critical factor in determining the likelihood of microbial growth, which only occurs in the aqueous phase.

In some products, the brine concentration can lie outside the 3 to 6% range. It is higher in some products, such as certain dry-cured cuts and dried sausages, which typically have a brine concentration of 13 to 16%. In some products, such as farmer salami, the concentrations can be as high as 30% (International Commission on Microbial Specifications for Foods, 1980, p. 388). In certain dry-cured cuts, the slow penetration of the curing agents may lead to extremes in concentrations within one product (International Commission on Microbial Specifications for Foods, 1980, p. 384).

At the 3 to 6% level in brine, sodium chloride alone would be inadequate to inhibit the production of C. botulinum toxin; however, in combination with other factors, sodium chloride is an important

1976). In certain products, such as some country hams and certain dried sausage products, C. botulinum can be completely inhibited by high brine concentrations. Cultures of Group I C. botulinum⁴ are able to grow and produce toxin in the presence of 8 to 10% brine, whereas cultures of Group II are inhibited by 5 to 6% brine (Genigeorgis and Riemann, 1979; International Commission on Microbial Specification for Foods, 1980, pp. 136-159).

pH. A pH range of 4.6 to 5.0 may limit the outgrowth of C. botulinum spores in certain media; however, the pH of most meats cured in the United States ranges from 5.5 to 6.6. On rare occasions, it may be as high as 7.0 to 7.2 (International Commission on Microbial Specifications for Foods, 1980, pp. 333-409). Reducing the pH from 7.0 to 6.0 or 5.5 lowered the salt tolerance of C. botulinum vegetative cells (Baird-Parker and Freame, 1967), and the nitrite sensitivity of Staphylococcus aureus has been observed to increase as pH was reduced from 6.9 to 5.5 (Castellani and Niven, 1955). As discussed below, residual nitrite appears to be important in the inhibition of C. botulinum (Christiansen, 1980; Christiansen et al., 1978). Thus, when considering manipulation of pH as a means of enhancing antimicrobial activity, one must recognize that the rate at which residual nitrite is depleted increases as the pH decreases (Nordin, 1969).

Direct acidulation of meat emulsion to inhibit microbial growth may affect the stability of the emulsion during handling, e.g., when stuffed into casings. Therefore, glucono- δ -lactone, which slowly hydrolyzes to gluconic acid, is often used to produce delayed acidulation (International Commission on Microbial Specifications for Foods, 1980, pp. 136-159).

Carbohydrates. Under abuse conditions, e.g., temperatures of 15-25°C, naturally occurring lactic-acid-producing bacteria in raw and perishable cooked cured meats will metabolize added or naturally occurring carbohydrates, thereby reducing the pH of the product by acid production, if sufficient fermentable carbohydrate is present (Christiansen et al., 1975). Protection by this mechanism undoubtedly explains observations in a recent study of bacon from four processing plants inoculated with C. botulinum spores (U.S. Department of Agriculture, 1979). One set of bacon samples contained 0.57% added sucrose; other sets of samples contained low concentration

⁴Group I contains proteolytic C. botulinum strains producing toxin types A, B, and F; Group II contains nonproteolytic C. botulinum strains producing toxin types E, B, and F. Group I strains have a

toxic. Within the first 28 days of the incubation, the pH of the sucrose-containing bacon was reduced to pH 5.0 or lower. The pH of bacon containing the lower concentration of sugar remained high (pH 5.4 or above) throughout the entire 56 days.

Storage Temperature. Temperatures of less than 10°C will inhibit outgrowth of spores of proteolytic C. botulinum strains producing type A or B toxins (Ohye and Scott, 1953). For example, no toxic samples were detected in perishable canned comminuted cured meat or in bacon incubated at 7°C (Christiansen et al., 1973, 1974). At temperatures below the optimum for C. botulinum growth (i.e., ~37°C) cell multiplication decreases with drops in temperature. This may in part explain the observation of Roberts et al. (1976) that the number of toxic samples observed after incubation at 15°C was less than that observed at 17°C.

Unfortunately, appropriate storage temperatures during distribution in retail stores, or in the home cannot be guaranteed, but the addition of curing salts can inhibit C. botulinum more readily as the storage temperature is reduced (Ingram, 1974; Roberts et al., 1976). Since the level of residual nitrite appears to be an important factor in promoting product safety (Christiansen, 1980; Christiansen et al., 1978), one should realize that nitrite depletion occurs more quickly when the product is subjected to temperature abuse (27°C) than when it is refrigerated (Christiansen et al., 1974). The implications of the depletion of these residuals are discussed later in this chapter.

Even if proper refrigeration could be assured, psychrotrophic strains of C. botulinum (capable of growth at 3.3 to 5.6°C) can multiply at usual refrigeration temperatures and could be a potential hazard (Eklund et al., 1967; Roberts and Hobbs, 1968). However, these strains are more sensitive to salt (Genigeorgis and Riemann, 1979) and are not common in red meats and poultry in the United States (Holley, 1978, 1981).

Water Activity. C. botulinum spore outgrowth, vegetative cell growth, and toxin production are inhibited at a water activity less than 0.93. If other environmental factors (e.g., pH and temperature) are not favorable, inhibition may occur at water activity levels higher than 0.93. The inhibitory effect of water activity is also influenced by the selection of solute. For example, strains of C. botulinum producing toxin types A, B, or E multiplied at lower levels of water activity when the media were adjusted with glycerol instead of salt (Baird-Parker and Freame, 1967). The water activity of most cured meats is higher than 0.95; however, certain raw, dry, fermented sausages have a water activity of 0.92 and a pH of 5.0 -- a combination

that prevents growth. In other products, the water activity may also prevent growth, e.g., when the brine concentration is approximately 10%, which is equivalent to a water activity of 0.92 (International Commission on Microbial Specifications for Foods, 1980, pp. 333-409).

Ascorbate or Isoascorbate (Erythorbate). In the United States, bacon is now formulated with 550 mg/kg sodium ascorbate or isoascorbate to accelerate the curing reactions and inhibit the formation of nitrosamines during cooking. Many other products are also formulated with ascorbate or isoascorbate (Tompkin et al., 1978a), but amounts added are not standardized in cured meats other than bacon. At 200 mg/kg, sodium ascorbate or isoascorbate enhanced nitrite's inhibition of C. botulinum in perishable canned cured meats, when the product was abused at 27°C shortly after manufacture (Tompkin et al., 1978a). However, higher concentrations (>500 mg/kg) in perishable canned cured meats decreased the effectiveness of nitrite (Tompkin et al., 1979b). The enhancement may have been due to the ability of ascorbate to chelate iron (Tompkin et al., 1978b,c, 1979a), because other sequestering agents, such as EDTA (ethylenediaminetetraacetic acid) or cysteine, also enhance inhibition by nitrite, and the addition of iron decreases inhibition. The levels of ascorbate or isoascorbate enhancing or reducing the effectiveness of nitrite in other types of products may not be the same as those for perishable canned cured meat. These effects need further investigation.

Ascorbate or isoascorbate should be used in cured meats only with great care because high levels may enhance the rate of nitrite depletion, thereby reducing protection against botulism (Tompkin et al., 1979b).

Added and Residual Nitrite Levels. There has been some disagreement as to whether the protection against botulism conferred by nitrite in a cured product can be predicted most accurately from the level of nitrite added or from the residual level of nitrite present at the time of abuse. Predictions are further complicated by the reactions of added nitrite with thermally processed meat. Some investigators have suggested that these reactions may result in the formation of inhibitory "Perigotype factors" (International Commission on Microbial Specifications for Foods, 1980, pp. 136-159; Pivnick and Chang, 1973), but the evidence for such factors is not conclusive (Sofos et al., 1979a). Moreover, temperatures exceeding 90°C sensitize spores to the inhibitory effects of nitrite and other curing salts, thereby further impeding a facile solution of these problems (Jarvis et al., 1976). Thus, the relative importance of added versus residual

Greenberg (1972) reported that for pasteurized canned hams, in which the level of heating does not damage spores, the likelihood of botulinum toxin production during temperature abuse could be predicted most accurately from the initial nitrite addition rather than from residual nitrite concentrations. Christiansen et al. (1978) studied the residual nitrite depletion and the germination, death, and outgrowth of botulinal spores in a canned, perishable ham product subjected to temperature abuse at 27°C immediately after processing with various levels of nitrite. They concluded that the safety of such products was dependent upon the presence of sufficient residual nitrite to inhibit germinated spore outgrowth until the number of viable cells had decreased to a point where cell growth could no longer be initiated.

They thus introduced the concept that the degree of protection depends on the outcome of a race between the depletion of nitrite (which is greater at abuse temperatures than under refrigeration) (Nordin, 1969) and the death rate of germinated spores (whose outgrowth is blocked by nitrite). During extended refrigeration of a product, residual nitrite may be reduced to a noninhibitory level, and viable, ungerminated spores may remain because of extended dormancy. If this product is then subjected to temperature abuse, the hazard of botulism increases since the remaining nitrite concentration is too small to be effective in controlling C. botulinum. A number of studies (cited earlier) have indicated that the protection provided by nitrite and other curing salt ingredients can be overwhelmed by high levels of inocula or contamination.

Christiansen (1980) presented data on nitrite depletion and C. botulinum death in hams at 27°C. This study emphasized that information on the rate at which viable C. botulinum cells decreased in number (which accompanies but may not be parallel to the rate of nitrite depletion, depending on temperature) is essential to determining the importance of residual nitrite.

These studies have been conducted with commercial products. However, there is no way of predicting if the levels of spore contamination introduced into these products and, thus, the numbers present at the time of temperature abuse, approximate contamination levels that might occur on occasion under normal commercial operating conditions.

The committee believes that the residual nitrite present at the time of abuse is one of the important determinants of the safety of cured products. Thus, any process or product changes that result in

Packaging. Product packaging may also affect microbial growth. During the early 1950s, a change from packaging films providing a low oxygen barrier to films with a high oxygen barrier was not accompanied by an observed increased incidence of botulism from cured meats, as had been feared. During the 1960s, vacuum packaging became widely used, but it was shown that, if other conditions were favorable, C. botulinum toxin would be formed whether the product was vacuum-packed or not (Christiansen and Foster, 1965). Vacuum packaging may delay growth of spoilage organisms and the production of typical spoilage odors, which often precede a potential microbial hazard. However, the presence of such odors is not a reliable indicator of toxin in foods (Christiansen and Foster, 1965; Sofos et al., 1980).

Curing Adjuncts. The addition of sodium tripolyphosphate and other polyphosphates to bind water or lessen shrinkage during cooking could increase the pH, thereby increasing the tolerance to sodium chloride of normal outgrowing C. botulinum spores, especially those injured by heating, and decreasing the inhibitory effectiveness of sodium nitrite. However, this increase in pH may be overshadowed by the ability of the negatively charged phosphate ions to chelate certain metallic ions, e.g., those of iron. Thus, polyphosphates may enhance the inhibitory effect of nitrite (Crowther et al., 1977; Skovgaard, 1980).

Efficacy of Nitrite as an Antibotulinal Agent

Tables 3-5 and 3-6 present some results of studies of the protection provided by nitrite against toxin production or swelling (spoilage) induced by C. botulinum in various inoculated commercial products. The fewer the days to toxin production or swelling for any nitrite-free product, the lower is the control provided by inhibitory factors (e.g., pH, salt, and water activity) other than nitrite. The lower the ratio of days to toxin production for nitrite-containing versus nitrite-free products, the smaller is the degree of protection provided by that level of nitrite. Nitrite protection against toxin production in fish has been demonstrated in chub, salmon, whitefish, and carp (M. W. Eklund, National Marine Fisheries Service, Seattle, personal communication, 1981). Levels providing protection will be discussed in the second report of the committee. For all product types shown in Tables 3-5 and 3-6, it appears that any reduction in added nitrite to levels lower than those currently

TABLE 3-5

Delay by Nitrite of Toxin Production in Bacon Inoculated with C. Botulinum Spores at an Abuse Temperature of 27°C.^a

Inoculum Spores/g ^c	Cure Ingredients ^b		Salt, %	Days to Comparable Toxin Pro- duction, Ratio No Nitrite to:			Samples Toxic At Time of Comparison, %	Days to First Toxin Pro- duction, Ratio No Nitrite to:			Referen-
	Sugar, mg/kg	Phosphate, mg/kg		30 mg/kg	60 mg/kg	120 mg/kg		30 mg/kg	60 mg/kg	120 mg/kg	
000	600	3,060	1.47	-- ^d	--	5:12	50	--	--	4:6	U.S. De Agric
000	20	750	1.69	--	--	10:15	40	--	--	2:3	U.S. De Agric
000	1,230	1,210	1.81	--	--	6:16	20	--	--	2:5	U.S. De Agric
000	4,975	4,640	1.40	--	--	15:>60	20	--	--	7:16	U.S. De Agric
000	8,000	5,000	1.70	--	--	13:>60	20	--	--	10:>60 ^f	Sofo s e Agric
000	8,000	5,000	1.70	--	--	<10:>40	20	--	--	<10:>40	Sofo s e
52	3,100	2,600	1.33	~10:~10	~10:~20	~10:56	5	<7:<7	<7:>7	<7:>40	Christi 1974

1.1 tests were conducted with bacon produced under commercial procedures, except that of Christiansen et al. 974), who used bacon prepared in a pilot plant. The efficacy of various treatments is analyzed by comparing the number of days of incubation that were required for the first package in a treatment group or for a particular percentage of those packages to become toxic at the simulated abuse temperature (27°C).

Concentrations listed were target values. Actual values may deviate, sometimes considerably from target values, e.g., U.S. Department of Agriculture, 1979.

Data in this column represent the lowest spore inoculum used in the cited studies in order to approximate most easily probable contamination.

Denotes not tested or not recorded.

Data pertain to toxic swollen packages from Phase 1 of the four-plant study conducted by the U.S. Department of Agriculture (1979).

The package became toxic at 16 days, none of the other 199 packages became toxic during the 60 days of the study.

TABLE 3-6

Delay of Nitrite of Toxin Production or Swelling in Various Commercial Cured Products Inoculated with C. Botulinum Spores at Abuse Temperatures^a

Inoculum, Spores/g ^b	Processing ^c	Sodium Chloride %	Days to Comparable Toxin Production or Swelling, Ratio of No Nitrite to:					Samples Toxic or Swollen at Time of Comparison, %	References
			40 mg/kg	50 mg/kg	100 mg/kg	150 mg/kg	156 mg/kg	200 mg/kg	
325	71°C	2.6	-- ^d	<14:<56	--	--	<14:>56	--	Hustad et al., 1977
5	--	--	--	< 7:<6	<7:>12	--	< 7:>12	--	Christiansen et al 1977
500	68.5	2.5	< 4:<4	--	--	--	< 4:<6	--	Sofos et al., 1979
100	68.5	2.5	--	7:43	7:102	--	7:107	--	Fumpkin, 1978; Tom et al., 1977
100	F ₀ = 0.4	2.3	--	--	10:14	--	--	10:17	Pivnick and Chang, 1973
100	58.3°C	2.5	--	<14:<14	--	<14:<21	--	--	Christiansen et al 1975
5	68.3°C	1.07	--	< 3:<6	<3:<10	--	< 3:<12	--	Christiansen et al 1977

Various treatments is analyzed by comparing the number of days of incubation that were required percentage of products in a treatment group to become toxic or swollen at the simulated abuse temperatures. The incubation temperature was 27°C in all studies except that of Pivnick and Chang 35°C.

^a represent the lowest spore inoculum used in the cited studies, in order to most closely represent contamination.

^b temperature.

^c tested or not recorded.

^d for products formulated without glucose and starter culture. For products to which glucose was added, but no nitrite, had been added, 10% of the samples were toxic within 112 days. For products and sodium nitrite (50 mg/kg), but no starter culture, had been added, toxin production was tested as long as 112 days.

time that the product could withstand temperature abuse without becoming toxic if contaminated, unless other inhibitory factors were appropriately modified. This will also be discussed further in the committee's second report.

The Contribution of Nitrite to the Control of Microbial Pathogens Other Than C. Botulinum in Cured Meats

Staphylococcus aureus is capable of growth and can produce its enterotoxin, under aerobic conditions, at salt concentrations greater than the 3% to 6% present in the aqueous phase of most cured meats (International Commission on Microbial Specifications for Foods, 1980, pp. 136-159, 333-409). Crowther et al. (1977) observed that it grew in bacon at 15-25°C, irrespective of the presence of sodium nitrite in concentrations up to 200 mg/kg, but the production of enterotoxin did not occur under anaerobic conditions.

Genigeorgis and Riemann (1979) reviewed information on the interaction of nitrite and other factors in controlling growth of S. aureus. They concluded that the levels of salt, nitrite, and the pH of most cured meats that are not dried extensively would not prevent growth or production of enterotoxin under aerobic conditions but that these factors may, in combination, become inhibitory under anaerobic conditions such as those that occur in vacuum-packed products. The committee concurs with this conclusion. However, the opportunity for the production of the heat-stable toxin still exists in products that are not vacuum-packed and in fermented and dried sausages during the fermentation and drying periods. This is apparent from the food-borne disease outbreaks described by Bryan (1980).

Salmonellae are not generally inhibited by the concentrations of salt and nitrite or by the pH of most cured meats. They are more resistant to nitrite than are C. botulinum cells, but, like S. aureus, they are much more sensitive to heat than are C. botulinum spores.

Gough and Alford (1965) have shown that sodium nitrite at 400 mg/liter or 6% sodium chloride were required to inhibit the growth of various strains of Clostridium perfringens in a thioglycollate medium. There appears to be little information on the effects of various combinations of nitrite, sodium chloride, pH, and water activity on the growth of C. perfringens in meat products.

Foodborne pathogens such as S. aureus, salmonellae, C. perfringens and mesophilic strains of C. botulinum are unable to multiply at refrigeration temperatures lower than 5 to 6°C, but psychrotrophic strains of C. botulinum (Group II) may grow at lower temperatures.

The Contribution of Nitrite to the Control of Microbial Spoilage of Meats

Table 3-4 lists some types of microbial spoilage that occur in certain raw and cured meat products. The spoilage pattern of a product will vary with the temperature at which it is stored (or abused). Spoilage and the factors involved in its control have also been reviewed by the International Commission on Microbial Specifications for Foods (1980, pp. 333-409). Since curing has successfully eliminated or greatly reduced many types of spoilage, there has been little motivation for conducting systematic investigations of the relative contributions to spoilage inhibition made by components of the curing salt, of the characteristics of the product (e.g., water activity and pH), or of the responsible organisms in various types of products. Many of the microbial strains involved in spoilage have not yet been satisfactorily categorized (International Commission on Microbial Specifications for Foods, 1980, p. 351).

In raw meats stored at temperatures below 10°C, Pseudomonas and the related Acinetobacter and Moraxella genera are particularly important spoilage agents (International Commission on Microbial Specifications for Foods, 1980, pp. 333-409) because they produce volatile amines, hydrogen sulfide, esters of organic acids, and slime. These organisms developed under anaerobic conditions, but they are succeeded, as the residual oxygen dwindles, by Microbacterium thermosphactum and Enterobacteriaceae. Ultimately, gram-positive organisms such as lactic-acid-producing bacteria predominate and eventually cause souring by acid production (International Commission on Microbial Specifications for Foods, 1980, pp. 333-409).

The addition of relatively low levels of salt (approximately 2%) to meat under aerobic conditions inhibits the growth of pseudomonads, resulting in the predominance of lactic-acid-producing bacteria. In raw cured meats, the initial flora is primarily gram-negative, but there is a subsequent shift to a predominantly gram-positive flora, e.g., micrococci, lactobacilli, streptococci, and Microbacterium thermosphactum (Giolitti et al., 1971; International Commission on Microbial Specifications for Foods, 1980, pp. 333-409). As noted above, salt contributes to this shift. At 200 mg/kg, sodium nitrite inhibits, to varying degrees, the growth of gram-negative organisms such as Pseudomonas, Achromobacter, Moraxella, Flavobacterium, Aerobacter, Escherichia, and some micrococci at pH 5.7 to 6.0 (Tarr, 1941a,b, 1942, 1944).

In dry-cured hams, spoilage is caused primarily by the growth of clostridia near the bone (Girolitti et al., 1971; Mundt and Kitchen, 1951). The data on C. botulinum control in other raw cured products presented in Table 3-5 and the work of Stumbo et al. (1945a,b,c) and Nordin et al. (1975) indicate that nitrite probably contributes to the control of the clostridia-induced spoilage in these products.

Spoilage of shelf-stable cured meat products and its control have been thoroughly studied by Stumbo et al. (1945a,b,c) and Nordin et al. (1975). The latter group of investigators derived from a model meat system a formula expressing the contribution of salt, pH, and nitrite to the control of spoilage. They studied cured meat heated to 115°C for 30 minutes within the pH range of 5.5 to 6.6. The salt content of the samples ranged from 1.5% to 3.0%, and concentrations of sodium nitrite ranged from 0 to 200 mg/kg. Within these ranges, an increase of 65 mg of sodium nitrite per kilogram of product provided inhibition equivalent to that produced by a decrease of 0.1 pH unit or a 0.3% increase in salt. The effects were additive. Thus, on a weight basis, additions of sodium nitrite were approximately 45-fold more inhibitory to spoilage than was salt. Caution must be exercised when using this model to make quantitative predictions of the effect of nitrite in commercial products or other types of cured meat because the model may not mirror their characteristics.

Various chemical tests have been suggested to quantitate spoilage, but none has gained widespread acceptance. Most of these tests provide only equivocal results at stages before the meat is obviously organoleptically spoiled (International Commission on Microbial Specifications for Foods, 1980, pp. 333-409).

Data on nitrite's contribution to the control of all spoilage organisms cannot be presented in a fashion similar to that shown in Tables 3-5 and 3-6 for C. botulinum in various products. Most evidence pertains to the inhibitory action of nitrite against spoilage clostridia (International Commission on Microbial Specifications for Foods, 1980, pp. 136-159, 333-409). This action is similar to the antibotulinal activity of nitrite. Many other types of organisms are also involved in spoilage, and it is not possible to define the precise point at which meat is spoiled based on numbers of any one species or on total numbers. Moreover, there are differences in the individual perception of spoilage (e.g.,

oxidation and color fading.

The Mechanism of Action of Nitrite

Studies in laboratory media indicate that nitrite, at the levels used commercially, inhibits the outgrowth of spores rather than their germination (Duncan and Foster, 1968; Gould, 1964; Pivnick, 1970). However, it is difficult to determine the mechanism by which nitrite inhibits microorganisms or spore outgrowth in products since the nitrite ion is capable of a variety of reactions in a given meat system and because such products are complex and lack uniformity. Benedict (1980) reviewed several possible mechanisms, namely, that nitrite (1) reacts with other components during heating to form an inhibitory substance; (2) acts as either an oxidant or a reductant on such cellular sites as enzymes, enzyme cofactors, nucleic acids, and cellular membranes; (3) reacts with cellular iron, thereby interfering with metabolism and repair mechanisms; or (4) reacts with thiols to form nitrosothiols, which in turn react with a spore membrane component, thereby interfering with metabolic and/or transport steps. In a bacteriological medium (containing tryptone, thioglycollate, nitrite, and iron), a substance (the Perigo factor) that inhibits C. botulinum is formed during heating. This factor is of questionable significance in perishable cured meats since it is formed at temperatures of 105°C or higher, which exceed those normally used in the processing of cured meats, and its antibacterial activity is neutralized by meat particles. Sofos et al. (1979a) have reviewed studies on the possible role of Perigo-type factors in cured meats (Pivnick and Chang, 1973).

Various studies offer clues to nitrite's possible mechanism of action. When nitrite is added to previously sterilized laboratory media, the inhibitory property of nitrite is enhanced by decreasing the pH to 6 and below. Such results suggest that free nitrous acid either is or produces the effective inhibitor. Metal ion-sequestering agents such as sodium ascorbate, EDTA, and cysteine enhance the antibotulinal effect of nitrite. Tompkin et al. (1978c, 1979a) suggested that nitrite reacts with the iron in ferredoxin, thereby rendering the latter inactive. Nitrite could also react with thiols and unsaturated lipids to form inhibitory oxidants. Because C. botulinum cells lack catalase and superoxide dismutase, they are sensitive to molecular oxygen and oxygen radicals.

Rowe et al. (1979) demonstrated that nitrite inhibits active transport, oxygen uptake, and oxidative phosphorylation by Pseudomonas aeruginosa, possibly by oxidizing ferrous iron of an electron carrier,

Support for the suggestion by Tompkin et al. (1978c) that ferredoxin is inactivated by nitrite was presented by Woods et al. (1981). They reported that nitrite inhibited the growth of Clostridium sporogenes cells by inhibiting the microorganism's phosphoroclastic system. This inhibition resulted from the reaction of nitric oxide, derived from nitrite, with the non-heme iron of pyruvate:ferredoxin oxidoreductase. Evidence that this mechanism also occurs in growing C. botulinum cells has recently been reported by Woods and Wood (in press).

The reasons that certain microorganisms are not susceptible to inhibition by nitrite have, unfortunately, received little attention. Page and Solberg (1979) investigated the exclusion of nitrite by cell-wall lipopolysaccharide and the existence of nitrite-metabolizing systems as possible resistance mechanisms. They concluded that the latter was the more likely reason for the relative lack of effect of nitrite on salmonellae as compared to susceptible microorganisms. Staphylococcus aureus also appears to metabolize nitrite when grown aerobically but not under anaerobic conditions (Buchanan and Solberg, 1972).

In summary, nitrite apparently attacks a number of targets in different bacterial species, and interference with the action of iron-containing enzymes may be a feature common to these inhibiting effects. It remains to be determined whether these effects are the primary mechanisms of action in complex meat systems and whether they are the mechanisms inhibiting clostridial spore outgrowth. Knowledge of the precise mechanism(s) of action of nitrite against vegetative cells and spore outgrowth in meat would facilitate the search for alternatives.

Interactions Affecting the Antimicrobial Activity of Nitrite

The antimicrobial activity of nitrite can be influenced by a variety of factors as described above and by the International Commission on Microbial Specifications for Foods (1980, pp. 136-159), Roberts et al. (1981a,b,c), and by Sofos et al. (1979a).

The antimicrobial action of both nitrite and sodium chloride salt depends on pH as does resistance to heat, but the effect of pH on heat resistance is relatively small in the pH range of 6.0 to 8.0 (International Commission on Microbial Specifications for Foods, 1980, pp. 1-37). The efficacy of nitrite correlates well with the undissociated nitrous acid concentration; a decrease of one pH unit

of nitrite needed for a given degree of inhibition. Differences in salt tolerances among bacterial species are magnified as the pH decreases from 7.0 to 5.5. Redox potential was shown by Castellani and Niven (1955) and Henry et al. (1954) to affect the antimicrobial activity of nitrite against some, but not all, species. Different bacterial species and strains, including strains of C. botulinum, vary in their resistance to the inhibitory effects of nitrite (Perigo and Roberts, 1968; Roberts and Garcia, 1973).

Incubation temperature and processing involving heat both affect the efficacy of nitrite. At abuse temperatures of 17.5°C, the inhibition of C. botulinum toxin production is less than at 15°C (Roberts et al., 1976). This may result from the increased rate at which residual nitrite is depleted as storage temperature increases or, more probably, because the higher temperature is more favorable to growth of mesophilic C. botulinum (Christiansen et al., 1974; Nordin, 1969). Pasteurization does not influence the susceptibility of spores to nitrite, but it eliminates certain potential competitors that germinating C. botulinum spores encounter in unheated products. Higher treatment temperatures damage spores, making them more susceptible to nitrite and sodium chloride (Pivnick, 1970; Roberts and Ingram, 1966; Roberts et al., 1966). Furthermore, nitrite, when heated, may react with meat components to produce inhibitory (Perigo-type) factors (Pivnick and Chang, 1973), but the evidence pertaining to this hypothesis is inconclusive (Sofos et al., 1979a).

Prediction of Control. Attempts have been made to develop models or equations to predict the interaction of factors contributing to microbial control and the effects on product safety to be expected if these factors were modified. These efforts have focused on protection against C. botulinum and other clostridia.

Pivnick and Petrasovits (1973) proposed the following formula for estimating the degree of protection against C. botulinum intoxication afforded by heat processing or curing salts in shelf-stable cured products:

$$Pr = Ds + In,$$

where Pr = protection, Ds is the destruction of spores due to heat processing, and In is the inhibition of spore outgrowth and cell multiplication by curing salts. In this equation, Pr, Ds, and In are measured in units of \log_{10} of the number of spores of C. botulinum. The formula can be also used for unheated products, in which case Ds = 0.

described by Nordin et al. (1975) as follows:

$$\hat{y} = -322 + 73.9 (\text{pH}) - 0.115 [\text{N}] - 24.7 [\text{S}],$$

where \hat{y} is the estimated percent of spoiled packages within 150 days at 23°C, pH ranges from 5.5 to 6.6, N is the concentration of sodium nitrite in the range 0 to 200 mg/kg, and S is the concentration of salt in the range of 1.5% to 3% (2% to 4% in the aqueous phase).

Both of these models are useful in describing the influence on safety provided by some of the factors involved in processing procedures.

Roberts et al. (1981a,b) have studied variables such as nitrite, nitrate, sodium chloride, isoascorbate, heat treatment, pH, and abuse temperature in a heated meat slurry model system. From their results they developed a computerized model that can be used to predict the influence of changes in these variables on the likelihood of toxin production within the limits used in the test system (Roberts et al., 1981c).

The committee strongly endorses the view expressed by Roberts et al. (1981c) that such models can be very helpful in assessing the relative effects produced by changes in concentrations of additives or other factors, but it believes that they should not be regarded as a means of providing quantitative predictions of changes in microbial proliferation in commercial products. There are no methods for predicting contamination levels, the probability and duration of temperature abuse, or variations in meat or product composition.

Other Factors Affecting Microorganisms in Cured Products

Within the United States, calculations of the amount of nitrite added to cured products are based on the uncured meat portion of the formulation. When extenders (such as soy protein) and other ingredients (such as previously cured products, especially those with low residual nitrite) are included in a product, the level of nitrite in the complete formulation will be less than that added to the meat.

Skovgaard (1980) has suggested that improved hygiene might justify lowering the level of nitrite added to products, but concluded that it was not yet feasible to determine the reduction that might be possible. The committee acknowledges that improved hygiene might lead to a lower probability of contamination with C. botulinum spores; however, it cautions that this reduction would be accompanied by a

range of bacterial species, but the mechanisms of this action are not fully understood. In some bacteria, they probably involve iron-containing enzymes. The most important antimicrobial effect of nitrite is its action against the putrefactive and pathogenic clostridia including C. botulinum. At the levels currently used in cured meat products, nitrite has no effect on germination, but it delays the outgrowth of spores of these organisms, thereby preventing spoilage and prolonging the period of temperature abuse that a cured product contaminated with C. botulinum can withstand before it becomes toxic (Tables 3-5 and 3-6). In this way, nitrite provides protection against the risk to health posed by botulism.

Depending on a number of factors, including the concentration of nitrite, environmental conditions, and the type of food product, nitrite may also contribute to the control of pathogens other than C. botulinum, including staphylococci, Bacillus cereus, and C. perfringens, and other spoilage organisms, such as bacilli, corynebacteria, and psychrotrophs, predominantly pseudomonads. The antimicrobial effects of nitrite are summarized in Table 3-7 at the conclusion of this chapter.

Relative Microbial Risks from Different Products. Realization of any potential microbial hazard to health posed by a cured product is obviously dependent on contamination of the product with a pathogen. But more important is the extent to which the production process, characteristics, and handling of the product allow multiplication of the pathogen. Factors that control the growth of the pathogens of most concern in cured meat products (e.g., C. botulinum, S. aureus, and salmonellae) vary among product classes. For some of these classes, factors other than nitrite may provide substantial protection. During the production of some products there may be opportunities for the multiplication of pathogens, e.g., staphylococci in fermented sausages if manufacturing practices are not optimal (Bryan, 1980). Thermal processing, final water activity, pH, and brine concentration in some products exert considerable control over some pathogens, in certain cases totally restricting their growth. Refrigeration is an effective means of controlling pathogen growth, but some products, e.g., shelf-stable canned cured meats, are not normally refrigerated. Thus if other controls fail, refrigeration does not play a part in ensuring their safety. Consumers show greater disregard for the need to refrigerate some perishable products, e.g., ham, than for others, e.g., frankfurters (Bryan, 1980).

to ensure their freedom from microbial hazard to health. The committee will present such an evaluation in its second report.

The extent to which inhibitory factors other than nitrite (e.g., brine concentrations) can be modified to provide protection against specific microorganisms is dependent on many considerations that may vary with different products. For example, increased salt might not be detectable in some meat products but may be unacceptable in fish. Additionally, the potential of such changes for producing adverse health effects, e.g., the influence of increased salt intake on hypertension, need evaluation. Such considerations will also be discussed in the second report.

ANTIOXIDANT EFFECTS OF NITRITE

Effects of Lipid Oxidation on Flavor

The fat (lipid) component of meat contributes substantially to its flavor, texture, and public acceptability. The basic meaty flavor resides in the water-soluble fraction of meat, but the flavor that distinguishes pork from beef or fish, for example, resides in the lipid fraction (Hornstein, 1967; Hornstein et al., 1960). Because lipids are sensitive to oxidative changes, it is not surprising that such changes can markedly affect flavor. For example, the rancidity that can develop in stored meat is due to lipid oxidation.

Tims and Watts (1958) discussed the rapid development of a rancid or stale flavor due to lipid oxidation that occurred in refrigerated cooked meats within 48 hours of storage at 4°C. They were the first to describe this as "warmed-over flavor" (WOF). In contrast to cooked meats, the onset of rancidity in raw meats, fatty tissues, rendered fat, or lard is usually much slower, not normally becoming apparent until these products have been stored for weeks or months (Pearson et al., 1977). However, Sato and Hegarty (1971) suggested that WOF also develops rapidly in raw meat that has been ground and exposed to the air, but the term has been most often applied to cooked meat.

To understand why and how meat becomes rancid, it will be helpful to discuss the two types of fat found in meat: storage (adipose) fat and structural fat. Storage fat is set aside in the animal for emergency energy needs and also for insulation of vital organs. This fat exists as globules within special fat cells of the adipose tissue, which is not part of the actual muscle structure.

The storage fat of animals consists mainly of saturated fatty acids, chemically stored as triglycerides. Saturated fatty acids are not oxidizable under the conditions of storage likely to be encountered during distribution and in retail stores. Storage fat also contains oxidizable monounsaturated fatty acids, mainly oleic ($C_{18:1}$), and polyunsaturated fatty acids such as the diunsaturated linoleic ($C_{18:2}$) and the triunsaturated linolenic ($C_{18:3}$) acids. The types of fatty acid present will depend on the species. The greater the percentage of unsaturated fatty acids, especially those that are polyunsaturated, the greater the degree of susceptibility to oxidation. The polyunsaturated fatty acid contents of various products are lamb < beef < pork < chicken < turkey < fish (Pearson et al., 1977; Wilson et al., 1976). For example, certain species of fish are very difficult to store, even in the frozen state, without rancidity developing within a few days (Pearson et al., 1977).

The fatty acid composition of the adipose tissue of nonruminants i.e., swine and poultry, can be changed by altering the diet fed to the animals, because the fatty acids consumed by these animals are incorporated into the fat unaltered after ingestion. Current feeding practices generally favor the accumulation of a high percentage of unsaturated fatty acids in the adipose tissue of pork and poultry (Pearson et al., 1977; Wilson et al., 1976).

The second class of fat is structural. Each muscle cell of the meat is surrounded by a lipid-containing membrane. Membranous structures also criss-cross the interior of the cell. Membrane lipids are composed of the unsaturated fatty acids, linoleic and linolenic acids, and arachidonic and longer chain polyunsaturated fatty acids. They are all highly susceptible to oxidation. The fatty acids in membranes exist in phospholipid structures and are sometimes classified as such. The amount of membrane lipid is constant per unit muscle mass, whereas the amount of storage fat can vary enormously. Because of the high percentage of polyunsaturated fatty acids, membrane lipids are especially vulnerable to oxidation (Wilson et al., 1976).

Chemistry of Lipid Oxidation

Oxidation disrupts the double bonds in lipids forming peroxides, polymers, and a variety of breakdown products, including aldehydes, ketones, and short-chain fatty acids. These small volatile molecules are the main contributors to rancid odor and flavor.

Oxidation appears to be a chain reaction involving a free radical mechanism:

According to Lundberg (1962), this autocatalytic reaction is initiated when a labile hydrogen (H) is detached from the lipid molecule (LH), resulting in the production of a lipid free radical (L[•]) and a hydroxyl radical (OH[•]). Reaction of the lipid free radical with oxygen (O₂) yields a peroxy radical (LOO[•]). This radical removes a hydrogen from another lipid molecule, thereby propagating the entire system. Decomposition of the lipid peroxide (LOOH) species forms more free radicals, giving rise to further chain reactions.

Catalysis and Mechanism of Lipid Oxidation

Muscle tissue contains a considerable amount of iron bound to proteins. Myoglobin, which resembles hemoglobin, is an oxygen storage protein within the muscle cells. Muscle tissues also probably contain residues of hemoglobin from blood. In addition, cells contain cytochromes. All these proteins contain the prosthetic group, heme, which has an iron atom at its center. The iron atom by itself promotes autoxidation of fats, but the entire iron-heme molecule appears to participate in the mechanism.

Tarladgis (1961) noted that in methemoglobin, for example, this iron has five unpaired electrons, which create a strong magnetic field that favors free radical formation. He suggested that decomposition of hydroperoxides could be mediated through donation of an electron from the cloud of the porphyrin ring.

A general scheme for the acceleration of lipid oxidation has been proposed by Tappel (1962). The principal mechanism seems to be catalysis of the decomposition of lipid peroxides (LOOH) by heme to form a lipid radical (L[•]) and a heme radical. The heme radical removes a hydrogen atom from another lipid molecule (LH), regenerating heme and, at the same time, generating a new lipid radical (L[•]).

Tappel also suggested that a heme molecule could attack a lipid directly:



These schemes and equations are those of chemical theory. In meat, the interactions are probably vastly more complex. Oxidation in meat occurs more rapidly than one would expect from consideration of the theoretical chemistry. There is also evidence that heme can

cooked meat. Igene et al. (1979) further demonstrated that non-heme iron is released from the heme pigments by cooking or by treatment with hydrogen peroxide, thereby accelerating lipid oxidation. These results agreed with the report by Haurowitz et al. (1941) that the prooxidation effect of hemin or hemoglobin on linoleic and linolenic acid is due to release of inorganic iron. It is possible that other polyvalent cations could also be prooxidants in meat and play a role in warmed-over flavor (WOF). However, Sato and Hegarty (1971) demonstrated that cupric salts actually inhibited WOF, apparently by the reaction of free radicals with cupric ions. Thus, non-heme iron appears to be the major prooxidant in the development of WOF (Igene et al., 1979).

Antioxidant Role of Nitrite in Cured Meats

Nitrite has been shown to retard lipid oxidation or development of WOF in cooked meat and processed meat products. Sato and Hegarty (1971) were able to eliminate WOF in cooked ground beef by adding sodium nitrite at a concentration of 2,000 mg/kg of beef and inhibit WOF at a sodium nitrite concentration of 50 mg/kg, as indicated by 2-thiobarbituric acid (TBA) values, a measure of lipid oxidation. Fooladi et al. (1979) investigated the role and function of nitrite in preventing the development of WOF in cooked beef, pork, and chicken. Added sodium nitrite (156 mg/kg) effectively inhibited the development of WOF in the cooked meat, resulting in a twofold reduction in TBA values in beef and chicken and a fivefold reduction in pork. Sensory panel scores confirmed the protective effect of added nitrite in meat from all three species.

Using cooked hams, MacDonald et al. (1980b) studied the effects on lipid oxidation resulting from the addition of sodium nitrite at 50, 200, or 500 mg/kg, butylated hydroxytoluene (BHT), or citric acid. Their data indicate that there is a significant reduction in TBA values in pork cured with sodium nitrite. Treatment of meat samples with BHT and citric acid reduced TBA levels, but these compounds were not as effective as the lower concentration of sodium nitrite (50 mg/kg).

The role of nitrite in minimizing WOF in cooked cured meats is not yet thoroughly understood, although Pearson et al. (1977) suggested that nitrite may either stabilize the lipid components of the membranes or inhibit the natural prooxidants in the muscle. Zipser et al. (1964) reported that nitrite exerts its effect by chelating iron, thereby preventing it from catalyzing oxidation. This concept has been investigated by MacDonald et al. (1980a), who found that dialysis of an aqueous extract of pork removed a fraction largely responsible

addition of 2% EDTA chelated non-heme iron effectively, resulting in a significant reduction in lipid oxidation in cooked meat.

The antioxidant properties of other nitrite derivatives in meat have also been demonstrated. S-Nitrosocysteine, a compound generated during the curing of meat, has been shown to act as an antioxidant both in aqueous linoleate model systems and in ground cooked turkey meat (Kanner and Juven, 1980). Kanner et al. (1979) also reported antioxidant activity of nitric oxide myoglobin (NOMb) in linoleate and β -carotene-linoleate aqueous model systems. The specific antioxidant activity of NOMb was maintained, even in the presence of prooxidants such as heme proteins and lipoxygenase.

Health Implications of Lipid Oxidation

Malonaldehyde (OHCH_2CHO) is produced by the oxidation of polyunsaturated fatty acids. Interest in its possible effects on human health has been stimulated by reports that it is mutagenic (Mukai and Goldstein, 1976). Brooks and Klammerth (1968) reported that malonaldehyde may react with DNA, thereby providing a possible rationale for its mutagenic activity. However, Marnett and Tuttle (1980) suggested that the observed mutagenicity of malonaldehyde may be due in substantial part to impurities resulting from the method used to prepare it. According to Shamberger et al. (1974), malonaldehyde is carcinogenic in mouse skin, when it is dissolved in acetone and applied topically.

Malonaldehyde was also tested as a complete carcinogen in Swiss mice by Apaja (1980), who applied the compound dermally in methanol and administered it orally in drinking water. When the compound was given to the mice in drinking water, it induced toxic effects in the stomach, where there was destruction, inflammation, and fibrosis of the glandular mucosa. Results of the chronic studies showed no tumors that could be attributed to malonaldehyde. Apaja (1980) concluded that malonaldehyde is not a complete carcinogen for Swiss mice.

A recent survey of the malonaldehyde content of 96 samples of fresh and processed meat and fish indicated that 92% of the processed or cured meats and 38% of the fresh meat contained less than 1 mg/kg. Sixty percent of the fresh meat samples contained concentrations of malonaldehyde ranging from 1 to 6 mg/kg (Siu and Draper, 1978). Whether these reported concentrations of malonaldehyde in meats have significance for human health is unknown, but reports that this compound may be toxic emphasize the desirability of minimizing its formation

Concern about the implications of lipid oxidation products in meats has focused on changes in consumer acceptability and loss of marketability rather than on the health hazard that they could present. Nevertheless, lipid oxidation should be considered in the overall safety assessment of meat, and its occurrence should be minimized in consumer products. Nitrite added to meat products inhibits oxidation and is particularly important in comminuted products into which air may be incorporated during manufacture. Some evidence indicates that the antioxidant effect of nitrite is greatest in pork products. The efficacy of alternative antioxidants will be discussed in the second report of this committee.

EFFECTS OF NITRITE ON SENSORY PROPERTIES OF MEAT PRODUCTS: FLAVOR, COLOR, AND TEXTURE

Chemical Aspects of the Formation of Cured Meat Flavor

The flavor of meat is a complex combination of characteristics such as taste, odor (aroma), texture, and temperature (Lawrie, 1974). Among these, aroma is of special significance, which is discussed below. Some studies have provided important insights into the development of flavor, but the precise origin of flavor remains uncertain. Studies conducted during the last 20 years, indicate that flavor results from a combination of volatile compounds produced during the heating of meat (MacLeod and Coppock, 1976). Heating also releases precursors to flavor from fat structures and allows intimate mixing of fat-soluble and water-soluble components (Herz and Chang, 1970).

Meat flavor is derived from both water-soluble (Hornstein and Crowe, 1960; Lawrie, 1974) and fat-soluble (Sink, 1973) nonvolatile precursors. The water-soluble precursors are low molecular weight compounds, including glycoproteins, reducing sugars, amino acids, and their degradation products (Batzer et al., 1960, 1962; Hornstein and Crowe, 1964; Wasserman and Gray, 1965). Precursors to meat flavor per se are similar in all species, whereas the difference in flavor among species is associated with the lipid fraction (Hornstein et al., 1960).

According to Chang and Peterson (1977), precursors to flavor, which may be unique to a given type of meat, are leached out of the aqueous phase during cooking and stored in the fat. In support of this suggestion is the fact that refined animal fat cooked by itself does not produce characteristic meat flavors or aromas. However, lipids themselves are not responsible for the formation of the sulfur- and nitrogen-containing heterocyclic compounds present in the volatile fraction of cooked meat.

and Suppen, 1976). Aliphatic and aromatic hydrocarbons, saturated alcohols, carboxylic acids, esters, ethers, aldehydes, and ketones are probably not primary contributors to meat flavor, according to a report by Chang and Peterson (1977). However, these investigators suggested that lactones, acyclic sulfur-containing compounds (mercaptans and sulfides), nonaromatic nitrogen-, oxygen-, and sulfur-containing heterocyclic compounds (e.g., hydrofuranoids), and sulfur-, nitrogen-, and oxygen-containing aromatic heterocyclic compounds (pyrazines and thiophenes) are probably the main contributors to meat flavor.

Despite the fact that many compounds have been identified in meats, none have been shown to be responsible for the specific characteristic flavors of the various products (Chang and Peterson, 1977; Herz and Chang, 1970). Many of the volatile components thus far identified in cured meats have also been found in uncured meats. No specific component or components possess the characteristic "cured" flavor of cured meat.

There have been many sensory analyses of cured meat flavor (Gray et al., 1981), but relatively few reports on the chemical interactions of nitrite and meat constituents that influence flavor (Bailey and Swain, 1973; MacDougall et al., 1975). Although nitrite is closely associated with cured meat flavor, especially that of ham (discussed later in this chapter), the chemical changes responsible for the unique flavor are not entirely understood. As a result, many investigators have attempted to identify the volatile compounds produced during the curing of meat.

Ockerman et al. (1964) conducted the first major study in this area. They extracted volatile compounds from dry-cured hams by vacuum distillation, collected them in a series of cold traps, and analyzed them by gas chromatographic retention times and infrared spectroscopy. The investigators identified aldehydes, ketones, acids, bases, and sulfur compounds, all of which are known to contribute to uncured meat flavor (Landmann and Batzer, 1966). The contribution of carbonyl compounds to the flavor of uncured meats has also been reported by Hornstein and Crowe (1963), Jacobsen and Koehler (1963), and Sanderson et al. (1966).

Cross and Ziegler (1965) isolated volatile compounds from both cured and uncured ham. They reported that the volatile compounds (mainly aldehydes) were qualitatively similar, but that the concentrations of these compounds varied among the products. For example, concentrations of pentanal and hexanal were higher in the uncured product than in the cured product. These investigators also observed that the volatile compounds retained a characteristic cured ham aroma

ardless of whether they originated from cured or uncured ham. After similar treatment, volatile compounds extracted from cured and uncured chicken and beef also had an aroma similar to that of cured ham. These authors proposed that cured meat flavor was derived from nonglyceride precursors, rather than from carbonyl compounds. Chang and Peterson (1977) also believe that carbonyl compounds are not major contributors to the flavor.

Using a vacuum distillation system equipped with cold traps, Lillard and Ayres (1969) extracted carbonyl compounds (e.g., alkanals, alk-2-enals, alk-2,4-dienals, and ketones), alcohols, and esters from country cured hams. The odor of the distillate, which collected in the traps, was reminiscent of cured ham. However, many of these compounds have been identified in the volatile fractions from other cooked meats. In another comprehensive study of the volatile constituents from cured ham, Swain (1972) reported that the aroma from ether-extracted ham resembled that of boiled ham. He also found that gas chromatograms obtained by capillary columns indicated that volatile components from cured hams treated with and without nitrite were qualitatively similar but quantitatively different. Among the classes of compounds identified were acids, aldehydes, alcohols, furans, ketones, hydrocarbons, nitrogen and sulfur compounds, and aromatics. The formation of several of the higher molecular weight aldehydes ($>C_5$) appears to be retarded by nitrite.

Plotrowski et al. (1970) isolated and identified cured ham aromas by studying various extracts from hams. They also observed changes in the constituents of pork during curing, cooking, and smoking. A trained panel characterized aqueous extracts and diffusates of cured and uncured hams. The panelists also assessed the odor produced upon heating. Aqueous extraction of all types of hams isolated the precursors of basic meaty aroma, whereas components or precursors of cured and smoky aromas were extracted from hams with a mixture of chloroform-methanol (2:1, v/v). The volatile substances developed during the heating of ham diffusates and lipid extracts were analyzed by gas chromatography, which indicated that there were some variations among the patterns of volatile compounds from six types of hams. But no single component had a meaty or cured aroma. These investigators concluded that compounds of intermediate volatility may be important contributors to meat flavor as well as better indicators of the differences between cured and uncured hams.

Few studies have been conducted on volatile compounds extracted from bacon. Using gas chromatography, Mottram and Rhodes (1974) found no clear-cut difference in the patterns of peak retention times for extracts of volatile substances obtained from cured and

columns. Compounds have not generally been identified by mass spectrometry or other rigorous analytical methods.

There appears to be a general consensus that carbonyl compounds are involved in the difference detected between the aromas of cured and uncured meats, but that other, as yet unidentified, components may also contribute to cured flavor. There are few data pertaining to the chemical basis for any contribution nitrite might make to cured meat flavor. MacDougall et al. (1975) suggested that cured meat flavor is probably a composite sensation derived from the contributions of many different odiferous compounds. The development of more sophisticated gas chromatographic procedures for use in conjunction with identification methods, such as mass spectrometry, could provide the stimulus for further studies in this area.

Chemical Aspects of the Formation of Cured Meat Color

The bright appearance created by the oxymyoglobin in fresh red muscle and the reddish-pink hue of denatured nitrosylmyohemochrome in cured meat products are attributes recognized by the consumer. Although the color of a meat product does not necessarily predict good texture and flavor, the shopper tends to make such an association (Giddings, 1977a; Jeremiah et al., 1972).

The color of both fresh and cured meat products is attributable primarily to the hemoprotein pigment, myoglobin (Clydesdale and Francis, 1971; Fox, 1966; Giddings, 1974, 1977a,b; Govindarajan, 1973). Figure 3-2 portrays the dynamic equilibrium between the various forms of myoglobin and demonstrates that the resulting hue of a meat product is dependent upon the oxidation state of the heme iron in the pigment and the type of functional group of the sixth ligand of the iron (Fox, 1966). The color of raw or fresh muscle tissue, such as beef or pork, is due to the dark red pigment, myoglobin (Mb); the cherry-red pigment, oxymyoglobin (O_2Mb); and the brown pigment, metmyoglobin (MMb) (Clydesdale and Francis, 1971; Reith and Szakaly, 1967a). Many factors influence the stability of these pigments (Clydesdale and Francis, 1971; Fox, 1966; Giddings, 1977a, b), and it is well known that they are not at all stable when the muscle tissue is heated (Reith and Szakaly, 1967a). To obtain a more stable red pigment in heated commercial meat products, nitrite is added before heating. Biochemical reactions in the meat reduce the nitrite to nitric oxide and the heme iron in myoglobin to the ferrous state. The interaction of these two species results in the formation of nitric oxide myoglobin (NOMB), a bright red pigment. When the meat product is then heated, the protein portion of NOMB is denatured and a rather stable pigment is formed, namely nitric oxide myohemochrome

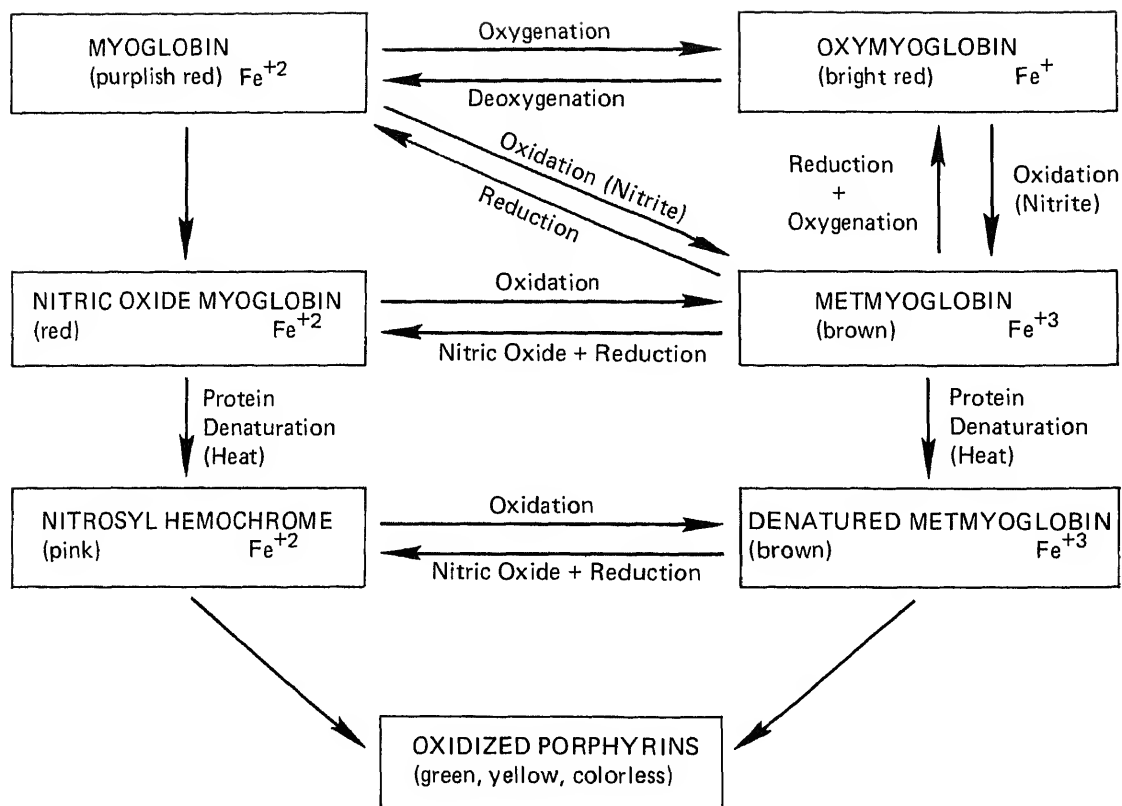
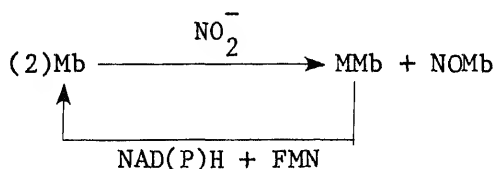


FIGURE 3-2. Some of the possible curing reactions that result from the addition of nitrite (Bard and Townsend, 1971).

1975; Reith and Szakaly, 1967a). Lee and Cassens (1976) reported that the amount of ^{15}N from labelled nitrite bound by heated samples of myoglobin was twice that bound by unheated samples. They postulated that the number of binding sites for nitric oxide doubled upon heating.

and myoglobin react under anaerobic conditions to produce NOMb and MMb (Giddings, 1977a). This implies that Mb can reduce nitrite to nitric oxide directly. In the presence of excess nitrite and a reducing system, the MMb formed is readily converted to the reduced form to participate again in the formation of nitric oxide and NOMb.

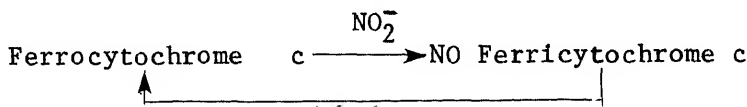
Several mechanisms have been hypothesized for the reduction of MMb in the presence of nitrite. The coenzyme systems, NADH (reduced nicotinamide adenine dinucleotide) or NADPH (reduced nicotinamide adenine dinucleotide phosphate) plus FMN (flavin mononucleotide), were studied by Koizumi and Brown (1971), who observed that these systems readily produced NOMb from MMb. This mechanism for the formation of NOMb can be shown as follows:

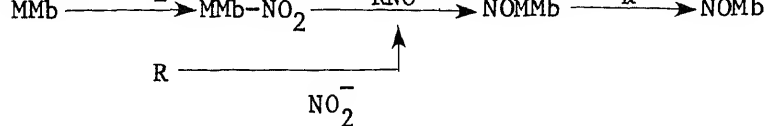


FAD (flavin adenine dinucleotide) and riboflavin can also promote the formation of NOMb by NADH, but nitrite was not reduced to nitric oxide in the absence of Mb.

Koizumi and Brown (1971) also studied a model enzyme-reducing system, diaphorase-methylene blue-NADH, which was effective in the formation of NOMb, but did not reduce nitrite in the absence of Mb. This proposed mechanism differs radically from the chemical scheme or enzymatic reactions proposed by others.

Walters and coworkers studied the participation of endogenous enzymes of the mitochondria in the reduction mechanism (Walters and Taylor, 1963; Walters et al., 1967). They observed that the formation of NOMb can proceed via nitric oxide metmyoglobin (NOMMb) by the transfer of nitric oxide from nitric oxide ferricytochrome c to MMb. Nitric oxide ferricytochrome c is formed from the reaction of nitrite and ferrocytochrome c. The NOMMb formed is then reduced to NOMb by NADH dehydrogenase. The cytochrome system is recycled by an NADH dehydrogenase. This system can be summarized as follows:





In this scheme, R represents a naturally occurring or added reducing compound. The scheme does not rely on enzymes, but, rather involves coenzymes and/or other reducing compounds, such as cysteine, hydroquinone, or ascorbic acid (Fox and Ackerman, 1968). This mechanism explains how color formation can occur before and after the product is cooked, whereas heating would eliminate the possibility of enzyme-coupled reductions, which were suggested by Koizumi and Brown (1971) and by Walters and Taylor (1963). Because ascorbic acid has been reported to be a very effective reductant in promoting color formation, it is usually incorporated into cure formulations to accelerate the development and increase the stability of cured meat color (Fox, 1966; Fox et al., 1967; Watts and Lehman, 1965).

All of these hypothesized mechanisms must be considered in light of the numerous factors that influence the rate and extent of NOMB formation in model and meat systems. Such factors include the type and/or relative concentrations of exogenous reductants, pH, the presence of salt/metal ions, storage temperature, level of nitrite added, temperature reached during heating or cooking processes, exclusion of oxygen during formulation, and the original pigment concentration in the meat (Acton et al., 1979; Fox et al., 1967; Giddey, 1966; Reith and Szakaly, 1967a,b; Renner and Rougie, 1979; Siedler and Schweigert, 1959; Watts and Lehmann, 1965; Weiss et al., 1953).

Once formed, the complex of myoglobin and nitric oxide is very stable in the absence of oxygen. In the presence of oxygen, which rapidly oxidizes free nitric oxide to nitrite, the stability of the complex is limited by the rate of nitric oxide dissociation since oxygen does not react directly with the bound nitric oxide (Giddings, 1977a,b). This dissociation rate is generally low, occurring by autoxidation in air (Walsh and Rose, 1956), oxidation by nitrous acid (Walsh and Rose, 1956), lipid-peroxide-induced oxidation (Younathan and Watts, 1959), or by photocatalyzed oxidation (Bailey et al., 1964; Walsh and Rose, 1956). The underlying principle for all such mechanisms of nitric oxide-heme dissociation is believed to involve the withdrawal of electron density from iron to porphyrin, which weakens the Fe-NO bond. The nitric oxide group dissociates, leaving the iron susceptible to oxidation by the "electronegative groups"

present in the medium (Tarladgis, 1962a,b). Such color loss is believed to be delayed by providing stronger reducing conditions in the medium (Bailey et al., 1964; Lin et al., 1980; Reith and Szakaly, 1967a; Tarladgis, 1962a,b), by incorporating nitrite exceeding the Mb level (Reith and Szakaly, 1967a,b; Tarladgis, 1962a,b; Walsh and Rose, 1956), by preventing exposure to energy-generating electronic excitation, e.g., light (Tarladgis, 1962a,b; Walsh and Rose, 1956), by replacing NO-based curing salts with nitrogenous compounds possessing strong electron-donor power (Siedler and Schweigert, 1959; Tarladgis, 1962a,b), by eliminating oxygen during storage (Fox, 1966; Reith and Szakaly, 1967b), by using packaging films with low oxygen permeability ($7 \text{ cm}^3/\text{m}^2/24 \text{ hr bar}$) combined with a maximum initial vacuum level of 687 to 737 mm Hg (Kraft and Ayres, 1954; Lin and Sebranek, 1979; Lin et al., 1980), and/or by increasing the pH of the product (Bailey et al., 1964; Reith and Szakaly, 1967a; Walsh and Rose, 1956).

The level of nitrite necessary to produce an "acceptable" color in various cured meats is discussed below.

Chemical Aspects of Cured Meat Texture

The reaction of nitrite with the non-heme proteins of meat probably alters the texture of the meat. However, this association is difficult to prove since measurements of the reactions with the protein are much more sensitive than the relative crude measurements used for the meat's texture. Moreover, the salt in cured meat also plays a major role in determining the physical properties of the product. Randall and Voisey (1977) concluded that nitrite did not affect the texture of ham and frankfurters.

Sensory Evaluation of Cured Meat Flavor

Although we typically speak of "tasting" flavors in foods, smell is the sense most involved in the identification of flavors (Mozell et al., 1969). The only qualities universally accepted as being discernible by taste are sweetness, saltiness, sourness, and bitterness. On the other hand, through the sense of smell we perceive such a complex array of qualities that no universally accepted classification scheme has been developed (Cain, 1978). When food is placed in the mouth, volatile compounds ascend the rear of the oral cavity to the olfactory mucosa, thereby effectively stimulating the sense of smell.

In 1940, Brooks et al. suggested that sodium nitrite produces a characteristic "cured" flavor. Subsequent studies on the sensory

Discriminative methods of sensory evaluation deal with the ability to detect differences. For example, the classic triangle test is a standard test in food technology in which a subject attempts to choose the odd sample from a group of three in which two samples are identical but the third differs. If a subject can choose the odd sample more often than would occur by chance, the odd sample is perceived to be different. However, this test does not indicate how the samples differ.

Descriptive methods of sensory evaluation permit an assessment of the way in which substances differ. The critical part of these methods is the choice of the attribute(s) to be described. For example, lemonade and milk have different tastes. There are many sensory qualities on which the two substances could be compared (e.g., sweetness and sourness). The selection of the relevant attributes is complex when foods are compared. The classic solution to this problem has been to train panels to recognize specific characteristics.

Scaling is also used in sensory evaluation. Once the attributes have been selected, one can simply ask for discriminative judgments (e.g., which sample is sweeter?), or the selected attribute can be quantified with a scaling procedure. The most common scaling procedures in use with foods are category scales. These scales are called "ordinal" scales because they can only order stimuli. For example, a person might be asked to assign the numbers 1 through 9 to a series of salt solutions on the basis that "1" means "weak," "5" means "medium," and "9" means "very strong." Ordinal scales express only order; they do not reflect the size of differences. For example, on a 9-point ordinal scale, a solution with a value of "4" is not necessarily twice as salty as one with a scale value of "2." The "4" indicates only that the solution is saltier than the one numbered "2."

When the size of the difference is important, then ratio scales are appropriate. One of the most commonly used is "magnitude estimation." With this method, subjects assign numbers that are proportional to the perceived intensities of the stimuli. In this scale, a value of "4" would reflect a stimulus with an intensity twice as great as one producing a value of "2."

Panel Selection. Panels used in the sensory evaluation of foods differ in their experience with foods and, possibly, with regard to sensory ability. Consumer panels are comprised of consumers with no special training beyond the specific instructions required by the task. Trained panel members do receive special training with food samples and may also be tested to ensure that their sensory abilities

group discussions to develop descriptive terms that can be adopted by all participants. An individual who consistently disagrees with the majority, or who cannot perceive attributes generally agreed upon, is often dropped from such a panel. Expert panels tend to be composed of individuals with extensive experience in tasting the food products of interest (Amerine et al., 1965, pp. 275-320). Obviously, the type of panel used for sensory evaluation will influence the results.

Individual Differences and the Search for Cured Meat Flavor.

Identical food samples do not have the same flavors for all individuals (Amoore, 1977; Bartoshuk, 1979). This interindividual variation in sensory perception may contribute to the variability observed in different sensory studies of cured meat flavor. Chemosensory sensitivity must influence studies of the ability to discriminate nitrite-cured from nitrite-free samples. If insensitive individuals are routinely eliminated from trained or expert panels, then these panels could be expected to detect differences more reliably than would a random sample of the population. This is acceptable when such a panel is used for quality control, but the results cannot be used to predict consumer behavior.

Methods Used in Studies on the Effect of Nitrite. Discriminative methods have commonly been used to determine whether or not nitrite-treated samples can be distinguished from controls.

Cho and Batzler (1970) were the first to use these methods systematically to study the contribution of nitrite to the flavor of cured meat. They used two sensory tests: the "triangle test," which tests panelists' ability to discriminate between samples, and the "two-sample test," in which panelists are asked to select the sample with more "cured flavor." The meat products used in these tests were pork roasts, i.e., porcine longissimus dorsi muscles. The panelists compared nitrite-cured and nitrite-free samples with variations in the amount of sodium chloride, the presence or absence of sugar, and smoking.

The comparison of a roast cured in a nitrite-containing pickle with its pairmate "cured" in distilled water produced the most dramatic results. In three replications of the triangle test, 14 of 23, 11 of 19, and 11 of 18 panelists correctly discriminated between nitrite-containing and nitrite-free samples, i.e., nine, eight, and seven panelists made errors. In three replications of the two-sample test, 19 of 23, 16 of 19, and 14 of 18 panelists judged that the nitrite-cured sample had the most "cured flavor." For this test, four, three, and four panelists selected a sample of pork roast "cured" in distilled water as having the most "cured flavor."

test provide statistically significant evidence for discrimination. That is, more panelists correctly distinguished between nitrite-cured and nitrite-free samples (in the triangle tests) and identified the nitrite-cured sample (in the two-sample tests) than would have been expected if panelists were selecting randomly. But why did any of the panelists make errors? There are two possibilities. Some individuals may be insensitive to the "cured flavor" imparted by nitrite, whereas others recognize it easily. Alternatively, the magnitude of the "cured flavor" imparted by nitrite may have been very small. One could easily determine which of these alternatives applied simply by noting whether or not specific individuals were selecting nitrite-containing samples reliably.

A number of studies using ordinal scaling methods have been conducted since Cho and Batzler reported their studies in 1970. These studies are discussed below in the section in which the contribution of nitrite to different products is assessed. Since ordinal methods only rank the cured flavor of one sample as greater than that of another sample, they do not indicate the magnitude of the difference. Thus, a study may indicate, in a statistically reliable sense, that a nitrite-cured meat has more cured flavor than a nitrite-free control, but the difference could be very small.

To the best of the committee's knowledge, no ratio scaling methods have been used to evaluate the magnitude of the contribution of nitrite to cured meat flavor. Nor was it able to find any reports of studies in which ratio scaling methods were used to assess the degree to which the flavors of two samples varied.

The color of the product can influence evaluations of cured flavor (DuBose et al., 1981). Thus, the color should be concealed when such a sensory evaluation is being conducted.

Contributions to Cured Meat Flavor by Factors Other Than Nitrite. In some products, cured meat flavor can be produced by sodium chloride alone (Greene and Price, 1975; MacDougall et al., 1975; Wasserman et al., 1977; Williams and Green, 1979). Smoking also plays an important role in cured meat flavor in some products (Wasserman and Talley, 1972). The generation of smoke may result in the simultaneous generation of nitrogen oxides, which are available for the production of color and flavor in the final product in a manner similar to that of added nitrite. The extent of such reactions is not known. Thus, the contribution of smoking to the overall color and flavor of cured meat products should be investigated further.

Because of the many variables in the type of meat product and processing, and because addition of the other flavoring agents e.g.

Principles of Chemoreception That are Relevant to the Perception of Cured Meat Flavor. As compounds in cured meats are identified, there is an understandable tendency to smell or taste them to determine if they are sources of cured meat flavor. The failure of this approach to identify a nitrite-specific flavor compound or compounds would not disprove that nitrite contributes to cured meat flavor.

"Cured meat flavor" is probably produced by a volatile compound or compounds and is discerned by the sense of smell, which appears to "synthesize" or "fuse" at least some aroma mixtures. For example, the aromas of two compounds may be perceived as one that is qualitatively distinct from either of the component aromas alone (Amerine et al., 1965, pp. 147- 148). Thus, "cured meat flavor" could be an aroma produced by a specific mixture of volatile compounds emitted by nitrite-cured meat. A compound-by-compound search might miss such an aroma mixture.

Hedonic Evaluation of Cured Meats

Hedonic Scaling. Rather than ask subjects to judge the sensory characteristics of meat samples, one can ask them to rate the product's appeal. They can either be asked to select the sample they prefer, or a scaling method (like those described for sensory tests) can be used to quantify how much a given sample is preferred. Both sensory and hedonic scales can be either ordinal or ratio scales.

Although hedonic testing asks the questions most pertinent to consumer preference, this method cannot easily produce answers to sensory questions. When asked to select the most preferred samples, consumers base their responses on color, flavor, texture, and probably several other criteria.

Other Measures of Preference. The behavior of consumers can be studied directly to provide information about acceptance. For example Williams and Greene (1979) studied the amount of uneaten nitrite-free and nitrite-containing bacon left on plates in order to determine acceptance. As in other hedonic testing, this method cannot easily produce information on the sensory characteristics of products.

Product-by-Product Evaluation of Nitrite and Cured Meat Flavor

Bacon. Bacon with an acceptable flavor can be prepared without nitrite. In studies with untrained panelists, Huhtanen et al. (1981) and Wasserman et al. (1977) found no preference differences between

Paquette et al. (1980) varied the amount of sodium nitrite in bacon samples from 0 to 120 mg/kg. Samples containing nitrite had a significantly more desirable flavor than did nitrite-free samples; however the desirabilities of the various samples with nitrite did not differ significantly, regardless of the concentration added. Although the nitrite-free bacon had a less desirable flavor than the nitrite-cured bacon, it was still acceptable.

Both the nitrite-free and the nitrite-cured bacon samples in the studies cited above contained sodium chloride. Kimoto et al. (1976a,b) reported that sodium chloride is more important than nitrite to the flavor of bacon produced in the United States. The importance of sodium chloride was also demonstrated by MacDougall et al. (1975) in studies of English (Wiltshire) bacon. They compared the bacon flavor of sodium-chloride-free and nitrite-free bacon as well as that of bacon cured with varying amounts of nitrite. The sodium-chloride-free samples had almost no bacon flavor, but the salted, nitrite-free bacon did.

Frankfurters. During the preparation of frankfurters, salt and nitrite are added to meat emulsions along with spices, sugars, and seasonings. Often, these products are also smoked.

Wasserman and Talley (1972) demonstrated that smoking is an important determinant of the flavor associated with frankfurters. Their panelists gave equivalent ratings of such flavor to nitrite-free and nitrite-cured samples when both were smoked. When unsmoked, the nitrite-cured frankfurters had more "frankfurter" flavor than the nitrite-free frankfurters.

Simon et al. (1973) found that all-beef frankfurters with no nitrite or with varying levels of nitrite were judged to have equivalent flavor quality, whereas the quality of half-pork, half-beef frankfurters varied greatly with nitrite level.

The contribution of sodium chloride to the flavor of frankfurters has not been evaluated. However, Greene and Price (1975) found that salt was the major contributor to cured meat flavor in samples of ground pork, whereas sodium nitrite alone produced very little cured meat flavor when used at a level of 200 mg/kg.

Ham. Brown et al. (1974), MacDonald et al. (1980c), and DuBose et al. (1981) confirmed the results of Greene and Price (1975) that sodium chloride can produce cured flavor. For example, MacDonald et al. (198 c) showed that "nitrite-free" ham samples containing salt possessed

Nitrite does make a contribution to cured flavor in hams cured in pickling solution. MacDonald et al. (1980c) cured hams with sodium nitrite levels of 50, 200, and 500 mg/kg. The lowest nitrite level, 50 mg/kg, was sufficient to produce a significant increase in cured meat flavor when compared to samples containing only salt.

DuBose et al. (1981) evaluated the influence of color in hams on flavor ratings. When color was concealed, low-nitrite samples were given higher ratings. When less red color was perceived in some samples, a lower rating was given to cured flavor. This "color-cueing" effect on flavor occurs at a level of nitrite that is lower than that currently used to cure ham.

Sensory Evaluation of Nitrite Levels Needed for Cured Color Formation

Only a small fraction of the nitrite added to a meat product is utilized for color fixation. Theoretically, only 3 mg of sodium nitrite per kilogram of product should provide a 50% conversion of Mb to NOMb (MacDougall et al., 1975). However, more is usually necessary to provide color stability because of the effects of the many above-mentioned factors, which influence the stability of the nitrosyl hemoprotein pigments, and also because of the reaction of nitrite with other meat components, such as sulfhydryl or amino groups (Cassens et al., 1974, 1979; Woolford and Cassens, 1977).

Kerr et al. (1926) noted that incomplete color formation resulted from insufficient nitrite penetration into the meat and/or unusually low myoglobin concentrations. This is exemplified by the fact that the minimum level of nitrite necessary to produce the desired color varies with the type of meat product, method of preparation, and presence of reductants such as ascorbate (MacDougall et al., 1975; Sofos et al., 1979a).

Using hedonic scales, Kemp et al. (1974, 1975) Eakes and Blumer (1975), and Eakes et al. (1975) reported that the application of sodium nitrite (at ≥ 250 mg/kg), potassium nitrate (up to 3,300 mg/kg), or their combination, to dry-cured hams resulted in color that was ranked more desirable (darker red) than the brownishgray hue observed in the salt and sugar-treated (control) sample. In both dry-cured hams and pork loins cured with sodium nitrite and/or potassium nitrate at 70-160 mg/kg, color was ranked significantly more acceptable when compared on a hedonic scale to that of products "cured" with no nitrate or nitrite (Eakes and Blumer, 1975). DuBose et al. (1981) reported that pickle-cured, smoked hams (open red with sodium nitrite at 25, 75, or 156 mg/kg

nitrite-free counterparts (Olson et al., 1979).

In comparison to pickle- or dry-cured products, comminuted meats require lower nitrite levels for color development because the chopping/emulsification process increases the available surface area and enhances the distribution of nitrite. Wasserman and Talley (1972) and Hustad et al. (1973) reported gray color in unsmoked frankfurters prepared without nitrite in the cure. Similar results were found in the sensory evaluation of salami sausage (Skjelkvale et al., 1974) and Thüringer sausage (Dethmers et al., 1975). Hustad et al. (1973) reported no significant difference in the color of frankfurters prepared with sodium nitrite concentrations of 50, 100, or 156 mg/kg. The lowest level of nitrite, plus smoking, imparted a characteristic color. Concentrations of sodium nitrite as low as 40 mg/kg resulted in acceptable color in chicken frankfurters (J. I. Gray, personal communication, 1981) and in turkey frankfurters (Sales et al., 1980), whereas 50 mg/kg was necessary for characteristic color to appear in a beef-pork bologna product (Lin and Sebranek, 1979) and in Thüringer sausage (Dethmers et al., 1975). In general, as the level of nitrite is increased, color acceptability of products increases (Sebranek et al., 1977). The colors, as indicated by Hunter color values, become more red and less yellow (Sales et al., 1980).

EFFECTS OF NITRATE IN CURED PRODUCTS

The committee found no evidence that nitrate had any direct effects in cured products, but believes that certain of its indirect effects, in addition to its capacity to yield nitrite by bacterial reduction, may have practical or sensory implications for such products as fermented sausages and dry-cured cuts.

Since nitrate is less reactive than nitrite (Chapter 4) and is not generally altered in foods, except by bacterial action, it can act as a reservoir from which nitrite can be produced over time. In certain cured products requiring long production times, e.g., fermented sausages and dry-cured cuts, it may be more practical to use nitrate than nitrite.

Certain species of bacteria, such as some micrococci (Buchanan and Gibbons, 1974), can reduce nitrate to nitrite. In the production of certain types of fermented sausage, especially European-style products (some of which are made in the United States), the availability of nitrate at the outset of the fermentation will promote the development

and possibly other contributors to flavor produced by these organisms in the subsequent fermentation and processing steps, are most probably different from those of the microflora that develops or is added (as starter culture) to products containing only nitrite. Thus, nitrate probably contributes indirectly to the traditional and characteristic flavor of these products (B. Tompkin, Swift and Co., personal communication, 1981).

Catalase, an enzyme produced by micrococci in the mixed flora, also appears to reduce peroxide development, thereby enhancing color stability and reducing the development of rancidity (Andres, 1977).

For products other than those noted above, the committee concludes that nitrite added at the minimum level necessary to achieve the desired effect could be substituted for nitrate.

The committee's findings on the effects of nitrate in fermented sausages and dry-cured cuts are based on limited information. The way in which nitrate acts in these and other products needs further investigation to substantiate conclusions discussed above.

SUMMARY: FINDINGS AND CONCLUSIONS

In the United States, nitrite and nitrate are added to cured red meats, poultry, and fish. The effects they exert depend upon the product to which they are added.

The committee found no evidence that nitrate had any direct effects in these products. However, it believes that certain of its indirect effects, in addition to its capacity to yield nitrite by bacterial reduction, may have practical or sensory implications for certain cured products, such as fermented sausages and dry-cured cuts.

Nitrite is used predominantly in cured meats. The motivation for its use is multifaceted. The effects of nitrite at current levels of use are shown in Table 3-7 for the major classes of cured red meats and poultry. The rankings of the relative importance of the various factors inhibiting spoilage microorganisms or pathogens are judgments of the committee based on data pertaining to U. S. products and, in a few cases, on data from studies in other countries. Additional references to the information in this table can be found in the applicable sections of this chapter.

Products ^a	Recommended Storage Temperature, °C ^b	Potential for Temperature Abuse or Contamination by Processor (P), Distributor (D), Consumer Before (CB) or After (CA) Opening	Spoilage Microorganisms		Inhibited by: ^c
			Reduced or Controlled by NO ₂ ⁻	Not, or Poorly, Controlled by NO ₂ ⁻	
Raw, Cured Products ^f					
High Water Activity:					
Bacon	< 4.4	D - Low CB - Low CA - Very low	Aerobic mesophilic Corynebacteria ^g	Lactics ^h Micrococci	Low temperature NaCl Anaerobic packaging NO ₂ ⁻ pH ²
Other pickled-cured products (e.g., smoked ham)	< 4.4	D - Low CB - Moderate CA - High	Clostridia and Bacilli	Psychrotrophs ^k Lactics ^h Micrococci	Heating ^f followed by low temperature NO ₂ ⁻ NaCl Anaerobic packaging pH
Low Water Activity:					
Dry-cured cuts (e.g., country ham)	0 to ambient (i.e., ~15-35)	D - Moderate ¹ CB - Moderate ¹ CA - Moderate ¹	Clostridia	Molds Yeasts	Low a _w NO ₂ ⁻ NaCl Anaerobic packaging
Dry, semidry, and fermented sausage (e.g., Lebanon bologna, salami)	0 to ambient (i.e., ~15-35)	P - Moderate CB - Low CA - Low		Molds Yeasts	Acid or low a _w Heating NO ₂ ⁻ Anaerobic packaging
Cooked, Cured Products					
Packaged After Heating: ^m					
Sausages (e.g., beef or chicken frankfurters) and some cold cuts	< 4.4	P or D - Low CB - Low CA - Very Low	Psychrotrophs ^k	Psychrotrophs ^k Lactics ^h Yeast	Heating followed by low temperature Anaerobic packaging
Canned:					
Perishable (e.g., canned ham)	< 4.4	D - Moderate CB - High CA - High	Clostridia and other putrefactive anaerobes	Some clostridia	Pasteurization with NO ₂ ⁻ followed by low temperature Sealed container NaCl
Shelf stable (e.g., luncheon meat)	Ambient (i.e., ~15-35)	P or D - Very Low CB - Extremely Low CA - Low	Clostridia, thermophiles		Thermal process Sealed container NO ₂ ⁻ NaCl
Commercially sterile (e.g., deviled ham)	Ambient (i.e., ~15-35)	P or D - Extremely Low CB - Negligible CA - Very Low	Spore-formers (with faulty processing)		Thermal process Sealed container (NO ₂ ⁻ /NaCl if processing faulty)

^aFurther examples of products of these categories can be found in the text (p. 3-6).

^bMost U.S. producers recommended < 4.4°C (40°F), but < 7.2°C (45°F) is still within some local laws.

^cAscorbate may potentiate the antimicrobial effects of nitrite, but is not effective by itself.

^dColor fixation by nitrite is selective for the muscle tissue of meat products.

^eReferences to nitrite's contribution to flavor in various product classes are as follows: bacon:

Huhtanen et al., 1981; Kimoto et al., 1976a,b; MacDougall et al., 1975; Paquette et al., 1980;

Wasserman et al., 1977; Williams and Greene, 1979; ham and ham-based products, including those

canned: Brown et al., 1974; DuRose et al., 1981; MacDonald et al., 1980c; dry-cured cuts:

Eakes and Blumer, 1975; Eakes et al., 1975; Kemp et al., 1974; fermented sausages: Dethmers

et al., 1975; frankfurters: Simon et al., 1973; Wasserman and Talley, 1972.

TABLE 3-7 (Continued)

Pathogens Reduced or Controlled by NO ₂ ⁻	Not or Poorly, Controlled by NO ₂ ⁻	Inhibited by ^c	Effects of Nitrite on:		
			Color ^d	Flavor ^e	Lipid Oxidation
<u>C. botulinum</u> <u>Staphylococci</u> ⁱ	Staphylococci ⁱ	Low temperature NO ₂ ⁻ Fermentable carbohydrate (if added) NaCl Frying ^j	Color fixation	Inconsequential contribution (salt major contributor)	Inhibits
<u>C. botulinum</u> <u>Staphylococci</u> ⁱ <u>B. cereus</u>	Staphylococci ⁱ Salmonellae	Low temperature NO ₂ ⁻ NaCl Anaerobic packaging ⁱ	Color fixation	Important contri- bution (salt also major contributor)	Inhibits
<u>C. botulinum</u>		Low a _w NO ₂ ⁻ NaCl Anaerobic packaging ⁱ	Color fixation	Inconsequential con- tribution (salt and lipid oxidation major contributors)	May limit a some oxida- has occur
<u>C. botulinum</u> <u>Staphylococci</u> ⁱ	Staphylococci ⁱ Salmonellae	Acid or low a _w	Color fixation	Important contribution (lactic acid produc- tion and salt also major contributors)	Inhibits
<u>C. botulinum</u>		Heating Low temperature NO ₂ ⁻ Fermentable carbohydrate (if added) Packaging NaCl	Color fixation	Important contribution, if product not smoked; NO ₂ ⁻ inconsequential, if smoked (spices also contribute)	Inhibits
<u>C. botulinum</u>		NO ₂ ⁻ Low temperature Heating Sealed container NaCl	Color fixation	Important contribution ⁿ	Inhibits
<u>C. botulinum</u>		Thermal process with NO ₂ ⁻ Sealed container	Color fixation	Important contribution ⁿ	Inhibits
<u>C. botulinum</u>		Thermal process Sealed container (NO ₂ /NaCl if processing faulty)	Color fixation	Important contribution ⁿ	Inhibits

^gSee text (p. 3-34). Dempster (1980) studied corynebacteria sensitive to heating at 63°C for 30 minutes.
^hLactics include lactic-acid-producing bacteria, "mainly atypical streptococci" whose speciation remains to be satisfactorily defined (Reuter, 1975).

ⁱSee text (p. 3-33); Gola and Cassolari, 1979; Labots, 1976.

^jFrying or other cooking by consumer or processor (as with prefried bacon).

^kPsychrotrophs could include gram-negative bacteria such as pseudomonads and coliforms as well as yeasts (see Terrell, 1974).

^lThis ranking is for packaged slices; for whole hams the potential is very low.

^mThus, subject to possible contamination during packaging.

Effects on Pathogenic and Spoilage Microorganisms

The specific contribution of nitrite to the inhibition of potential pathogens and spoilage microorganisms varies with the product in which it is used and with variations in their production, handling, and abuse.

Nitrite, in association with other components in the curing salt mix, exerts a concentration-dependent antimicrobial effect in cured products including, but not limited to, inhibition of the outgrowth of spores of putrefactive and pathogenic clostridia, including Clostridium botulinum. Nitrite thus provides protection against the risk to health posed by botulism. Under conditions of excessive contamination or prolonged temperature abuse, nitrite does not indefinitely prevent such outgrowth, and spoilage and/or toxin production may ultimately ensue.

Residual nitrite appears to be an important determinant of the degree of protection provided by nitrite. Thus, any product or process changes that result in a lower level of residual nitrite, e.g., adding less nitrite or increasing its rate of depletion, will increase the likelihood of the product becoming toxic if contaminated and abused. However, other factors that influence the risk of botulism e.g., contamination or the timing and duration of temperature abuse, are not predictable. Thus, it is not possible to derive a quantitative relationship between the protection provided by nitrite and the risk of botulism or to determine the degree of protection that is necessary to ensure the safety of a particular product. The committee believes that it is not practicable to produce raw meat, meat products, or fish products with a guarantee that they do not contain microbial contamination. Under these circumstances, the prudent approach to protecting the public health is to base precautions on the assumption that product contamination by pathogens and opportunities for their growth (temperature abuse) are both likely to occur.

Depending on a number of factors, including the concentration of nitrite, environmental conditions, and the type of food product, nitrite may also contribute to the control of pathogens other than C. botulinum, for example, Staphylococcus aureus, Bacillus cereus, and C. perfringens. Nitrite retards microbial spoilage of cured meats by inhibiting the growth of a variety of organisms, especially anaerobic and aerobic spore-forming bacteria, such as clostridia and bacilli.

growth of vegetative cells of certain microorganisms is not fully understood, but in some bacteria it appears to involve reaction with iron-containing enzymes. A more thorough knowledge of the mechanism would facilitate a search for alternative antimicrobial agents.

Special considerations are relevant to the use of nitrate and nitrite in fish products. Most important among these are the higher frequency of contamination of fish with C. botulinum and the fact that the most common contaminating strains are able to grow at lower temperatures.

Various cured products differ in the opportunities they present for microbial multiplication during production, in the extent to which control is exerted over pathogens by factors other than nitrite (e.g., pH, brine concentration, and thermal processing), and in the care taken during consumer handling. Based on these considerations, product types can be ranked according to the degree to which the addition of nitrite is desired to ensure their freedom from microbial hazard to health. The committee will present such an evaluation in its second report, which will discuss alternative approaches to the current use of nitrite.

Effects of Nitrite on Lipid Oxidation

Another preservative property of nitrite in cured meat products is its ability to minimize lipid oxidation, which yields products that cause rancidity and may be toxic. This effect of nitrite is particularly important to preserve flavor in comminuted products, but may not be necessary for this purpose in dry-cured cuts.

Effects of Nitrite on Sensory Characteristics

Nitrite produces a distinctive color in the muscle tissue of cured meats as a result of its reaction with myoglobin.

The contribution of nitrite to flavor has not yet been fully determined for all cured meat products. Because of differences among products, this contribution should be examined on a product-by-product basis. Nitrite appears to make a significant contribution to the flavor of pickle-cured hams and ham-based products.

Sodium chloride is largely responsible for the "cured" flavor in some products, especially bacon. Other product ingredients (e.g., spices) or processes (e.g., smoking) may also be important contributors to flavor. It is possible that flavor characteristics can not be

For reasons described in this chapter and also in Chapter 5, the committee recommends that:

Methods should be developed for the rapid and accurate determination of residual nitrite in meats at levels less than 10 mg/kg.

Research should be conducted to determine the potential of cured meats to nitrosate amino substrates in vivo. Furthermore, investigations should be conducted to determine the potential of the nitrogen-containing compounds arising from added nitrite (e.g., "residual" nitrite, nitrosothiols, nitric oxide myoglobin, nitrosated amides, peptide linkages, and nitrosated lipids) to act as nitrosating agents.

Antimicrobial Action

The mechanism(s) of action by which nitrite delays clostridial spore outgrowth and inhibits the growth of susceptible vegetative cells should be investigated further. The reasons that some bacterial groups are not susceptible to nitrite should also be determined. This work should be accorded high priority in view of its importance in developing alternatives to nitrite.

The contribution of nitrite to the control of pathogens other than C. botulinum in cured meats should also be determined. Moreover, further definition of the role of nitrite in controlling spoilage organisms in cured meats is desirable, especially for genera other than clostridia.

High priority should also be accorded to investigations of the interaction of factors controlling pathogens and spoilage organisms in different commercial products in order to develop methods for predicting the degree of control gained or lost through alteration of any of those factors. However, predictions from these models should be verified by testing under commercial conditions, before changes in production practices are introduced.

As a contribution to assessing the microbial hazard to health from cured products, surveys using serial sampling techniques should be conducted to determine the frequency with which C. botulinum spores contaminate different classes of raw meats and cured products under diverse production conditions and in different geographic locations.

The mechanism by which nitrite inhibits lipid oxidation in cured meat products should be investigated.

Sensory Studies

Psychophysical methods, with the capacity to determine the magnitude of any flavor differences, should be used to investigate the contribution of nitrite to the flavor of cured meats to determine if a dose-response relationship exists and what form it takes. Such studies should initially be conducted on ham because evidence indicates that nitrite contributes to the flavor of this product. The contributions of sugar, salt, and other potential contributors to flavor should also be investigated.

To derive the maximum potential from sensory studies, research should be conducted to determine the distribution of the ability to discriminate "cured meat flavor" in the general population.

Studies should be conducted to determine the extent to which nitrogen oxides from "smoke" contribute to color and flavor of meats that do not contain nitrite and to elucidate how smoking affects the overall sensory characteristics and other properties of meat.

Use of Nitrate

For products other than fermented sausages and dry-cured cuts, the committee recommends that nitrite added at the minimum level necessary to achieve the desired effect should be substituted for nitrate.

It also recommends that further investigation be conducted to determine the need for, and mode of action of, nitrate in fermented sausages and dry-cured cuts.

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THE CHEMISTRY OF NITRATE, NITRITE, AND NITROSATION

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Elemental nitrogen and oxygen combine in various proportions to form entities such as nitrate (NO_3^-), nitrite (NO_2^-), hyponitrite ($\text{N}_2\text{O}_2^{=}$), and several nitrogen oxides, including nitrous oxide (N_2O), nitric oxide (NO), dinitrogen trioxide or nitrous anhydride (N_2O_3), nitrogen dioxide (NO_2), dinitrogen tetroxide (N_2O_4), and dinitrogen pentoxide (N_2O_5). Both nitric oxide and nitrogen dioxide are relatively stable free radicals and are therefore sometimes denoted NO^\cdot and NO_2^\cdot , respectively.

This chapter is limited to the chemistry of those compounds which can directly or indirectly participate in nitrosation reactions, especially in the formation of N-nitroso compounds containing the group $>\text{N}-\text{N}=\text{O}$. These compounds are referred to in the text as nitroso derivatives of the parent amine compound without the prefix, e.g., nitrosodimethylamine, rather than N-nitrosodimethylamine.

NITRATE AND NITRITE

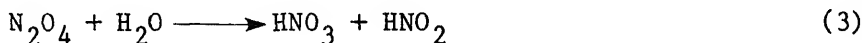
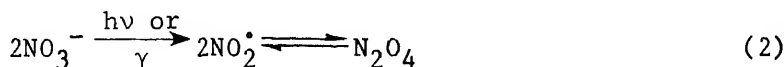
Nitrate salts of several metallic elements (MNO_3) are distributed widely in nature, whereas nitrite salts (MNO_2) are less common because of their higher chemical reactivity. The underlying reason for this difference is that nitric acid (HNO_3 ; $\text{pK}_a \sim 1.3$) is a stronger acid than nitrous acid (HNO_2 ; $\text{pK}_a 3.4$) and, therefore, forms less readily from its salts. Both the occurrence of nitrite and nitrate salts and exposure of humans to these compounds have been thoroughly reviewed (Archer, in press; Green et al., in press; National Academy of Sciences 1978; White, 1975, 1976). Estimates of current exposure are presented in Chapter 5.

Chemical Transformation

Nitrate salts are often hydrated, and nearly all are soluble and highly dissociated in aqueous media (Mellor, 1928). Both the salts and the nitrate ion are relatively chemically inert. The reactions that are most important in this discussion, i.e., the reduction of nitrate ion to either nitrogen dioxide or nitrite ion (NO_2^-), do not occur readily under neutral or mildly acidic conditions. Generally, such reactions require temperatures in excess of 500°C or treatment with a reducing agent such as iodide ion (I^-) or metals in the presence of strong acid (Mellor, 1928). The only transformations of potential biochemical interest appear to be the reduction of alkali metal nitrates to the corresponding nitrites by photolysis (Bayliss



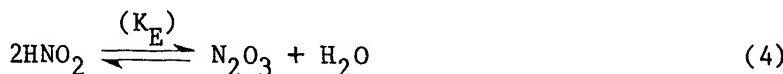
proceed via nitrogen dioxide, which hydrolyzes to a mixture of nitrous and nitric acids following dimerization to dinitrogen tetroxide (equations 2 and 3):



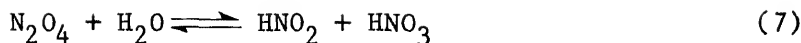
The reductive radiolysis proceeds at dose levels commonly used in the sterilization of foods by radiation, and the reductive photolysis can be induced under atmospheric conditions (Petriconi *et al.*, 1967). Significantly, dinitrogen tetroxide is a powerful nitrosating agent toward amino compounds in aqueous and other solvents (discussed below in the section on nitrosation by nitrogen oxides).

Nitrite salts are much more reactive than the corresponding nitrate salts because the nitrite ion is a stronger nucleophile than the nitrate ion (Edwards, 1954) and because nitrous acid is weaker than nitric acid. Thus, nitrous acid is generated under mildly acidic conditions ($\text{pH} < 5$) and much of the chemistry of nitrite salts relates to the presence of nitrous acid.

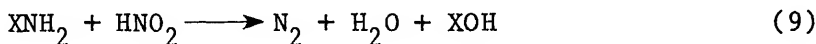
In aqueous solution, nitrous acid exists in equilibrium with nitrous anhydride (dinitrogen trioxide, N_2O_3), as shown in equation 4, where $K_E = 0.2$ at 20°C (Turney, 1960):



Dinitrogen trioxide is unstable and decomposes, especially when heated, to a mixture of nitric oxide and nitrogen dioxide (equation 5). Dimerization of nitrogen dioxide to dinitrogen tetroxide and subsequent hydrolysis (equations 6 and 7) produces a mixture of nitrous and nitric acids. Thus, after standing, aqueous acidic solutions ($\text{pH} < 5$) of nitrite salts tend to decompose to the nitrate salt, thereby losing nitrite activity.

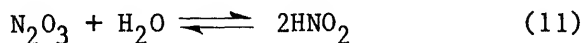
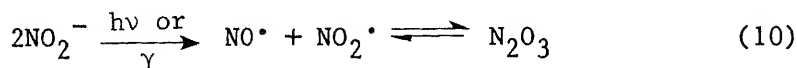


Nitrous acid (and, inter alia, nitrite salts) can be either oxidized to nitrate ion ($\text{HNO}_2 + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + 3\text{H}^+ + 2\varepsilon$; $E^\circ = -0.94 \text{ V}$) or reduced to nitric oxide ($\text{HNO}_2 + \text{H}^+ + \varepsilon \rightarrow \text{NO} + \text{H}_2\text{O}$; $E^\circ = -1.0 \text{ V}$). Oxidation of the acid requires relatively strong reagents such as manganese dioxide (MnO_2) or chlorine (Cl_2), but weaker oxidants are effective in alkaline media ($\text{NO}_2^- + 2\text{OH}^- \rightarrow \text{NO}_3^- + \text{H}_2\text{O} + 2\varepsilon$; $E^\circ = -0.01 \text{ V}$) and the spontaneous decomposition of nitrous acid (equation 8) also converts it to nitric acid. Nitrous acid is readily reduced to nitric oxide by reaction with a wide range of inorganic materials such as copper (Cu^+), iron (Fe^{2+}), iodide (I^-), and bisulfite (HSO_3^-) ions, and organic compounds such as ascorbic acid, polyphenols, and thiols. Thus, nitrous acid is a useful oxidant (Turney and Wright, 1959). The behavior of nitrous acid in the presence of reducing agents is not predictable from the reduction to nitric oxide alone. Other products may result, depending on the reductant selected, the acidity, and the temperature. Thus, reaction of nitrite ion with hydrogen sulfide (H_2S) yields nitric oxide and sulfur in acidic solution, ammonia (NH_3) and sulfur in bicarbonate buffer, and ammonia, sulfur, and thiosulfate ($\text{S}_2\text{O}_3^{2-}$) in unbuffered nitrite salt solutions (Moeller, 1952). Furthermore, nitrous acid is reduced to nitrogen in the presence of compounds containing primary amino groups, e.g., ammonia, primary amines (RNH_2) and amides (RCO.NH_2), hydrazine (H_2NNH_2), urea ($\text{H}_2\text{NCO.NH}_2$), sulfamic acid ($\text{NH}_2\text{SO}_3\text{H}$), or hydroxylamine (NH_2OH) (equation 9):



Aqueous solutions of alkali metal nitrites are also reduced by radiolysis (Dainton and Logan, 1965; Grätzel et al., 1970) and photolysis (Treinin and Hayon, 1970) in a manner analogous to the corresponding nitrates. The reactions differ, however, insofar as a mixture of

nitric oxide and nitrogen dioxide is obtained from nitrites (equations 10 and 11), but only nitrogen dioxide is obtained from nitrates.



Thus, there is no net loss of nitrite in a closed system, but generation of dinitrogen trioxide as an intermediate results in nitrosation reactions under nonacidic conditions. Many of the redox reactions of nitrous acid and nitrite salts are relevant to their biochemical behavior. They explain why nitrate salts exist at much higher concentrations than nitrite salts in the environment and the mechanisms by which many compounds inhibit the formation of N-nitroso compounds.

Both nitrous acid and, to a lesser extent, nitrite salts can nitrosate a variety of inorganic and organic materials. These reactions, which proceed via several reactive nitrosating agents, are discussed in detail below. They are relevant to biochemical processes, especially the formation of N-nitroso compounds. Furthermore, several of the redox processes in which nitrous acid is reduced either to nitric oxide or to nitrogen involve an initial nitrosation of the reductant.

Biological Transformation

Biological or enzymatic transformations of both nitrate and nitrite salts are well-known components of the nitrogen cycle. They are redox processes associated with both the conversion of atmospheric nitrogen to the nitrate-plant nutrient and its conversion to ammonia during assimilation by plants and microorganisms.

Much of our knowledge concerning the microbial oxidation of nitrogen comes from studies of the autotrophic microorganisms Nitrosomonas and Nitrobacter (Schmidt, 1978). Nitrosomonas catalyzes the oxidation of ammonia and hydroxylamine (NH_2OH) to nitrite, whereas Nitrobacter catalyzes the oxidation of nitrite to nitrate, which is relevant to the present discussion. This oxidation is known to involve a flavomolybdoprotein and cytochrome, which probably act sequentially. The activity is linked to the respiratory chain and is associated with the cell membrane (Nicholas, 1978). Some of the

with the oxidation of 1 mole of nitrite to nitrate (Kosaka et al., 1979; Parks et al., 1981).

In plants and microorganisms, nitrate is reduced to ammonia by two, well-defined enzymatic processes. In the first, nitrate is reduced to nitrite in a reaction that is catalyzed by the flavomolybdo-protein, nitrate reductase (EC 1.6.6.3). In the second, the reduction of nitrite to ammonia is catalyzed by the iron-containing protein, nitrite reductase (EC 1.6.6.4).

Nitrate reductases have been isolated from bacteria, fungi, algae, and plants. Their molybdenum (Mo) content is approximately one atom per mole, and they have a molecular weight of 1.6 to 6×10^5 daltons and a K_m for nitrate from 0.015 to 1 mM (Hewitt, 1975). In bacteria, two different nitrate reductases have been identified (Hewitt, 1974), and their properties are discussed in a review written by Green et al. (in press). Nitrate reductase activity has also been demonstrated in various rat tissues (Cohen and Weinhouse, 1971). Its properties are similar to those of xanthine oxidase, which can also reduce nitrate to nitrite (Rajagopalan et al., 1962). Nitrite reductase has also been isolated from bacteria, algae, fungi, and plants. This enzyme contains iron, has a molecular weight of 0.6 to 1.2×10^5 daltons (but 3.5 to 6.7×10^5 daltons for E. coli and yeast), and a K_m for nitrite of 0.005 to 0.07 mM (Hewitt, 1975). In plants and algae, enzymes are usually specific for donors of single electrons (ferredoxin, methyl viologen) and do not utilize reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Hucklesby and Hewitt, 1970). In yeast and bacteria, however, the enzyme is specific for NADPH and is flavin dependent (Prabhakararao and Nicholas, 1970).

E. coli also has two nitrite reductases, one of which is specific for reduced nicotinamide adenine dinucleotide (NADH) and is unable to reduce sulfite (SO_3^{2-}), whereas the other is specific for NADPH and can reduce sulfite (Abou-Jaoudé et al., 1979). An alternative mechanism has been proposed for nitrite reduction by bovine heart cytochrome c, whereby cyclic turnover by the cytochrome yields nitric oxide from nitrite ion and an electron donor (Orri and Shimada, 1978). As nitric oxide is produced during the anaerobic incubation of fresh pig muscle with nitrite ion at pH 6 (Walters and Taylor, 1964), this reaction may be general for muscle tissue.

There is good evidence that nitrate and nitrite salts are both formed and destroyed endogenously by microflora or by metabolic action. For example, nitrate ion is reduced to nitrite ion by microflora normally present in the mouth (Goaz and Biswell, 1961; Ishidate et al., 1974; Keith et al., 1930; Spiegelhalter et al.,

1978; Sander and Sell, 1980; Schrag et al., 1980; and by flora that inhabit the infected urinary tract or bladder (Cruickshank and Moyes, 1914; Guignard and Torrado, 1978; Jóhárt, 1978; Kumin and DeGroot, 1977; Scheifele and Smith, 1978; Sinaniotis et al., 1978).

Nitrate salts are also reduced by mammalian enzymes in vitro (Cohen and Weinhouse, 1971; Rajagopalan et al., 1962). As noted above, nitrate ion forms from nitrite ion via oxyhemoglobin (Kosaka et al., 1979) and, possibly, from more reduced nitrogen compounds by mammalian systems (Green et al., in press). These reactions, together with the metabolism, distribution, and excretion of nitrate and nitrite salts in animals and humans, have been discussed by Green et al. (in press) and Archer (in press).

NITROSATION

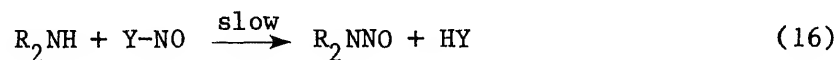
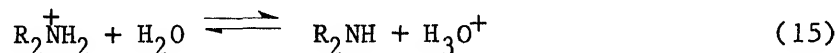
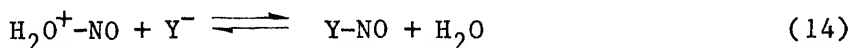
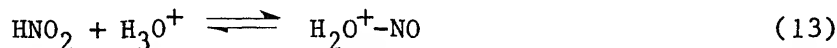
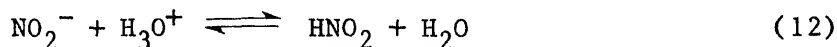
Nitrosation usually results from reaction of compounds that can generate a nitrosonium ion (NO^+) and, less often, nitric oxide (NO). Free nitrosonium ion can be obtained only in strong acids or in solid salts such as nitrosonium tetrafluoroborate (NO^+BF_4), but it is readily available from labile nitrosating agents (represented as YNO , where Y is a nucleophilic catalyst) generated in nitrite solutions and by gaseous nitrogen oxides. These nitrosating agents react with almost any nucleophile (i.e., electron-rich entity) such as amines, alcohols, and anionic species.

Reactions with amines and amides are of primary interest because they may produce carcinogenic compounds including nitrosamines [R(R')NNO] and nitrosamides [R(R'CO)NNO]. However, nitrosation of compounds in food and in vivo has to be considered in connection with catalytic and inhibitory effects. The formation of nitrosamines from nitric oxide is less common, but it does occur with amine anions (R_2N^-) and when oxidants generate either amino radicals ($\text{R}_2\text{N}^\bullet$) or radical cations ($\text{R}_2\text{N}^{\bullet+}$).

Carcinogenic N-nitroso products derive from many different amino compounds including most secondary (R_2NH) and tertiary (R_3N) amines, secondary and tertiary amides (RCO.NHR' , RCO.NR'R'), N-substituted ureas (R'HNCO.NH_2), guanidines [R'HNC(=NH)NH_2], and urethanes (RR'N.COOR). The most common N-nitroso compounds are derived from either secondary amines (RR'NH) or their N-acylated analogues [R(R'CO)NH].

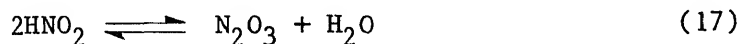
The chemistry of N-nitrosation in vitro is relatively well understood (Challis, 1981; Ridd, 1961). The principles can be applied to reactions in food and in vivo to explain both catalysis and inhibi-

Aqueous acidic solutions of nitrite salts (or nitrous acid) at pH < 5 are the best known nitrosating media, and their reactions have been widely investigated (Challis, 1981; Ridd, 1961). Neither nitrous acid nor nitrite ion reacts directly with the amino substrate. The effective nitrosating agent (Y-NO) forms from the nucleophilic catalyst (Y⁻, e.g., NO₂⁻, Cl⁻, SCN⁻) and protonated nitrous acid in a rapid, preequilibrium step (equations 12, 13, and 14).



Only the unprotonated amino substrate, which is in equilibrium with its conjugate acid (equation 15), reacts with Y-NO (equation 16). Thus, reaction rates are dependent on pH, the basicity of the amino substrate, and the presence of catalytic anions (Y⁻) in addition to the concentrations of amino substrate and nitrite ion.

In the absence of other nucleophiles, nitrite ion acts as the catalyst, Y⁻, in which case the reactive species is dinitrogen trioxide (nitrous anhydride) formed in equilibrium with nitrous acid (equation 17).



For basic secondary amines (pK_a > 5), the rates of nitrosamine formation calculated from the gross amounts of amine and nitrite salt added (equation 18) have a characteristic dependence on pH, reaching a maximum at approximately pH 3.4 and, for amino acids, at approximately pH 2.5 (Mirvish, 1975). This reflects the

$$\text{Rate} = k_1 [\text{amine}] [\text{nitrite}]^2 \quad (18)$$

counteracting effects of acidity on the concentrations of dinitrogen trioxide and unprotonated amine. The level of observed rates over the entire pH range, however, is dependent on the amine basicity (pK_a), which determines the proportion of unprotonated amine available for reaction. Data in Table 4-1 show clearly that N-nitrosamines form most rapidly (i.e., largest k_1 values) from the least basic amino substrates. However, rate coefficients k_2 in Table 4-1, calculated from the actual concentrations of nitrous acid and unprotonated amine in solution, have similar values irrespective of the amine pK_a .

$$\text{Rate} = k_2 [\text{R(R')NH}][\text{HNO}_2]^2 \quad (19)^1$$

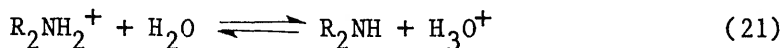
Thus, the reactivity of these compounds is probably governed by factors such as diffusion through the solvent and is not due solely to the ease with which the unprotonated amine attacks dinitrogen trioxide. This suggestion has important ramifications in explaining the effect of catalysts.

Reaction via dinitrogen trioxide seems to apply to most secondary alkyl and heterocyclic amines and amino acids at pH 2 to 5 in the absence of catalysts such as thiocyanate (SCN^-) or iodide (I^-) ions. It is important that neither the type nor concentration of buffer seems to have an appreciable influence, suggesting that similar rates may apply to reactions in gastric contents.

Weakly basic amino compounds (e.g., amides, ureas, and some aromatic amines) are too unreactive to combine readily with dinitrogen trioxide. At approximately pH 2 or lower, however, they undergo nitrosation by another pathway, which is attributed to a direct reaction of the neutral substrate with either hydrated or unhydrated nitrosonium ion, i.e., H_2ONO^+ or NO^+ (Challis, 1981; Mirvish, 1975; Ridd, 1961) (equation 20).

$$\text{Rate} = k_3 [\text{R(R')NH}][\text{HNO}_2][\text{H}_3\text{O}^+] \quad (20)$$

Equations 21 and 22 show acid-catalyzed nitrosation of a weakly basic amino compound by acidified nitrous acid. Usually, these reactions



Rate Constants k_1 and k_2 (Equations 18 and 19) for the Nitrosation of Amines at the Optimum pH and 25°C

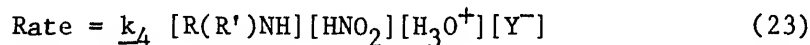
Amine	pK _a	Optimum pH	k_1 (M ⁻² sec ⁻¹)	$k_2 \times 10^{-5}$ (M ⁻² sec ⁻¹)	Reference
Piperidine	11.2	3.0	0.00045	1.4	Mirvish, 1972
Dimethylamine	10.72	3.4	0.0017	1.5	Mirvish, 1970
Pyrrolidine	11.27	3.0	0.0053	21.0	Mirvish, 1975
N-Methylethanol-amine	9.5	3.2	0.010	0.62	Mirvish, 1975
N-Methylbenzyl-amine	9.54	3.0	0.013	0.92	Mirvish, 1975
Proline	-	2.5	0.037	1.4	Mirvish <u>et al.</u> , 1973
Sarcosine	-	2.5	0.23	2.6	Mirvish <u>et al.</u> , 1973
Prolylglycine	8.97	3.0	0.25	5.0	Mirvish <u>et al.</u> , 1973
Hydroxyproline	-	2.5	0.31	2.1	Mirvish <u>et al.</u> , 1973
Prolylleucylglycin-amide	8.97	3.4	0.38	6.2	Mirvish <u>et al.</u> , 1973
Morpholine	8.7	3.4	0.42	2.3	Fan and Tannenba 1973; Mirvish, 1972
Mononitrosopiper-azine	6.8	3.0	6.7	0.38	Mirvish, 1972
Aminopyrine	5.04	2.0	80	1.0	Mirvish <u>et al.</u> , 1974
Piperazine	5.57	3.0	83	0.62	Mirvish, 1972
N-Methylaniline	9.8 4.85	-	250 ^a	18.0 ^a	Kalatzis and Rid 1966

^aCalculated from rates at pH 1 and 0°C.

increasing acidity. Furthermore, the reactivity of these substances is strongly dependent on their structure. This is shown in Table 4-2 for reaction at pH 2 and 25°C, where the fastest reactions (largest k_3) tend to apply to the most basic compounds (Mirvish, 1975).

To sum up this section, the rate of nitrosation of most secondary amines is proportional to the square of nitrite concentration (equation 19) and shows a maximum value at pH 3 to 3.4 (equation 19). The rate of nitrosation of N-alkylureas, N-alkylcarbamates, simple amides, and some aromatic amines is proportional to the nitrite and hydrogen ion concentrations. Thus, it does not show a pH maximum, but increases steadily as the pH drops (equation 20). Despite these differences, it can be stated that, in general, weakly basic secondary amines, N-alkylureas, and N-alkylcarbamates are most readily nitrosated, and that strongly basic secondary amines, simple N-alkylamides, and guanidines are nitrosated more slowly. Primary, tertiary, and quarternary amines usually yield nitrosamines still more slowly, with the exception of the tertiary amine compound aminopyrine, which is extremely rapidly nitrosated (Table 4-1). However, the relative ease of nitrosation will vary according to the conditions, especially the pH and the nitrite concentration, both of which affect reactions differently, as shown in equations 19 and 20 (pH below 3, and low nitrite concentrations favor the reaction shown in equation 20).

Catalysis. In the presence of anionic (Y^-) or nucleophilic entities (HY), nitrous acid forms additional $Y-NO$ reagents (equations 14 and 16) and nitrosamine formation follows equation 23:

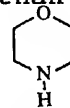


The formation of N-nitroso compounds is accelerated principally by raising the concentration of nitrosating agents (Fan and Tannenbaum, 1973; Williams, 1977). Some of these reagents may also be more reactive than dinitrogen trioxide. Examples of the $Y-NO$ species include nitrosyl thiocyanate ($SCN-NO$), which is formed from the thiocyanate ion, and nitrosyl iodide ($I-NO$), which is formed from the iodide ion. The rate of nitrosation according to equation (23) is proportional to the concentration of nitrite.

Strong accelerations by thiocyanate and iodide ions have attracted attention because of their possible relevance in vivo (Boyland and Walker, 1974; Boyland et al., 1971; Fan and Tannenbaum, 1973). In smokers, salivary thiocyanate ion levels are known to be elevated. Iodide ion is present in gastric secretions. The degree of catalysis

Amide	k_3 ($M^{-2}sec^{-1}$)	Nitrosamides: UV Absorption in Water [λ in nm (ϵ)]		Reference
N-Methylbenzamide	0.0014	406 (75)	Mirvish, 1975	
N-Methylacetamide	0.0025	402 (66)	Mirvish, 1975	
Dihydrothymine	0.0035	406 (95)	Mirvish, 1975	
Methylguanidine	0.004	-	Mirvish, 1971	
1-Methyl-3-nitroguanidine	0.008	420 (109)	Mirvish, 1975	
Dihydrouracil	0.010	406 (94)	Mirvish, 1975	
Hydantoin	0.042	417 (66)	Mirvish, 1972	
Ethyl N-ethylcarbamate	0.10	420 (64)	Mirvish, 1971	
Hydantoic acid	0.18	390 (85)	Mirvish, 1975	
Ethyl N-methylcarbamate	0.37	410 (71)	Mirvish, 1971	
2-Ureidoethanol	0.38	398 (89)	Mirvish, 1975	
DL-Citrulline	0.72	396 (83)	Mirvish, 1971	
Phenylurea	2.2	400 (74)	Mirvish, 1975	
Ethylurea	3.0	400 (78)	Mirvish, 1971	
Trimethylurea	7.0	400 (36)	Mirvish, 1975	
Ethylcyanamide	8.0	388 (126)	Mirvish, 1975	
Methylurea	10.5	400 (79)	Mirvish, 1971	
Ethoxyphenylurea	13	420 (49)	Mirvish, 1975	
2(1H)-Tetrahydropyrimidinone (propylene urea)	18	411 (96)	Mirvish, 1975	
1,3-Dimethylurea	200	400 (72)	Mirvish, 1975	
2-Imidazolidinone (ethylene urea)	400	400 (76)	Mirvish, 1975	

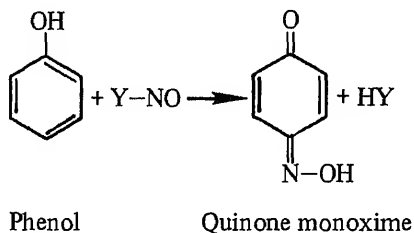
The accelerations for weakly basic secondary amines, e.g., N-methylaniline ($C_6H_5NHCH_3$), are greater (Fan and Tannenbaum, 1973) than those for more strongly basic secondary amines, e.g., morpholine



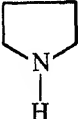
(Boyland and Walker, 1974; Boyland et al., 1971). No anion catalysis is observed for amides (Berry and Challis, 1974), thioamides ($RCSNHR^-$) (Al-Mallah et al., 1974), ureas, and urethanes (Hallett et al., 1980). Berry and Challis (1974) proposed a mechanism to explain this.

A number of electron-rich compounds other than amines react with acidified nitrite to yield Y-NO compounds, but examples leading to catalysis of nitrosamine formation by the Y-NO are rare. Usually the nitroso product Y-NO is either too stable to react further or so

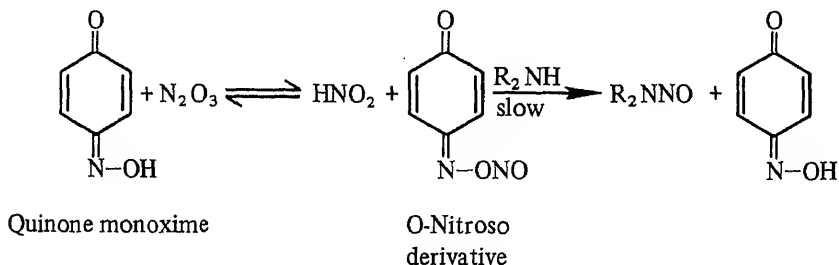
An interesting example of both is found with phenol (C_6H_5OH), which is known to be present in gastric secretions. Phenol reacts readily with aqueous nitrous acid at $pH < 4$ (equation 24) to give the stable quinone monoxime (Challis and Lawson, 1971):



Subsequently, it has been shown that quinone monoxime catalyzes

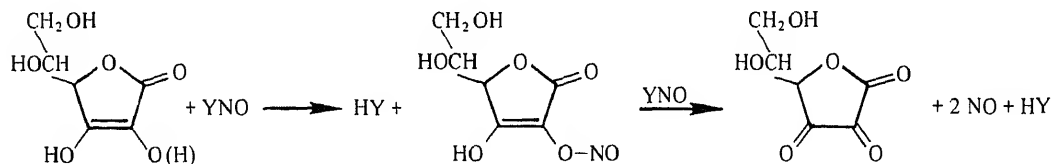
the nitrosation of pyrrolidine () (Davies and McWeeny,

1977) and diethylamine (Walker *et al.*, 1979), probably by rapid formation of an O-nitroso derivative, which then reacts with the secondary amine:

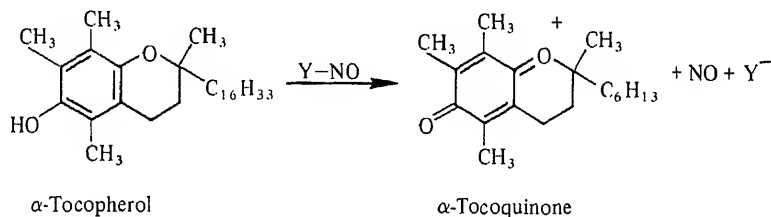


Under these circumstances, nitrosamine formation is proportional to [nitrite] rather than to [nitrite]². The pathway will be important at high [nitrite]/[phenol] ratios only. Other nucleophiles whose interactions with nitrous acid appear to catalyze nitrosamine formation are sulfur compounds and alkenes. Thus, nitrosation of dimethylamine [(CH₃)₂NH] at pH 4 is strongly catalyzed by thiourea [(NH₂)₂C=S] (Masui *et al.*, 1977):

sulfur dioxide (SO_2), bisulfite (HSO_2^-), ascorbic acid (vitamin C), tocopherols (e.g., vitamin E), 1,2- and 1,4-dihydroxyphenols [$\text{C}_6\text{H}_4(\text{OH})_2$], gallic acid [$\text{C}_6\text{H}_2(\text{OH})_3\text{COOH}$], and other well-established synthetic and natural "antioxidants." Their ability to inhibit the formation of N-nitroso compounds both in vitro and in vivo has been examined assiduously, as summarized in a recent review by Douglass et al. (1978). Mirvish et al. (in press) have championed the application of ascorbic acid as an inhibitor. It is effective over a wide range of pH because both the free acid and the ascorbate ion rapidly reduce Y-NO to nitric oxide (Dahn and Loewe, 1960):

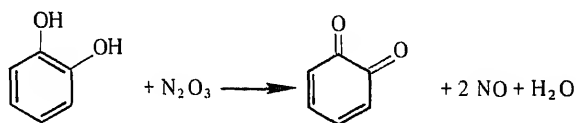


For lipophilic matrices, however, the lipid-soluble α -tocopherol may be superior (Fiddler et al., 1978; Mergens et al., 1978). This is discussed further in Chapter 6. α -Tocopherol acts like 1,2-dihydroxyphenols (see equations 28 and 29) in being oxidized by dinitrogen trioxide to a quinonoid product (Mirvish, 1981; Newmark and Mergens, in press).

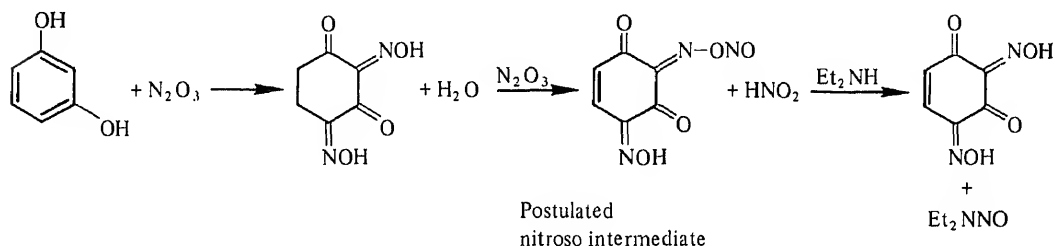


Other recent work has clarified confusing results concerning the effect of polyhydroxylated phenols on nitrosamine formation. In particular, Pignatelli et al. (1980) have shown that 1,2- and 1,4-dihydroxyphenols (including naturally occurring flavonols) inhibit nitrosamine formation at pH 4 and that earlier reports of catalysis

or artifacts. The inhibition results from reduction of the nitrosating agent, e.g., dinitrogen trioxide, to nitric oxide (NO):



1,3-Dihydroxyphenols (e.g., resorcinol) are powerful catalysts under similar conditions (Pignatelli *et al.*, 1980). This is attributed to rapid formation of a nitroso intermediate (equation 30), which interacts with more dinitrogen trioxide to generate a powerful nitrosating agent analogous to that proposed for catalysis by quinone monoxime (compare equation 25 with equation 30).



The reduction of nitrous acid to nitric oxide leads to inhibition because nitric oxide is an ineffectual nitrosating agent in the absence of catalysts. To be effective, however, it is necessary to add excess reducing agent because the ready oxidation of nitric oxide back to nitrogen dioxide and subsequent formation of dinitrogen trioxide ($NO + NO_2 \rightleftharpoons N_2O_3$) may quickly restore nitrosating capability. This effect has been noted for the formation of nitrosomorpholine in the presence of ascorbic acid (Archer *et al.*, 1975).

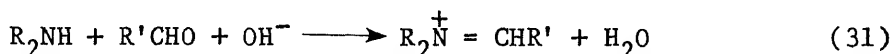
Production of nitric oxide can enhance nitrosation in a two-phase system, as has occurred inadvertently during the preparation of nitrosation mixtures (reviewed by Mirvish, 1981). Nitric oxide is extracted into the lipid phase and oxidized by oxygen to nitrogen dioxide. Lipid-soluble compounds, e.g., amines in an alkaline medium, can then be nitrosated in the lipid phase by the dinitrogen trioxide, which results from the reaction of nitric oxide and nitrogen dioxide. This system of nitrosation may be important in some industrial processes.

reductive methods discussed above. Pyrrole (, however,

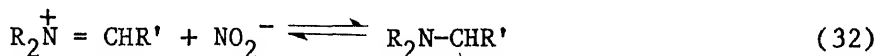
inhibits the formation of nitrosomorpholine (Groenen, 1976), and other reactive heteroaromatic compounds may act similarly.

Nitrosation by Nitrate and Nitrite Salts

Nitrosation by nitrite ion proceeds in the presence of certain carbonyl compounds, chlorinated solvents, metal salts, or radiation. Formaldehyde (HCHO), pyridoxal (C₈H₉NO₃), and benzaldehydes (RC₆H₄CHO), but not acetone (CH₃COCH₃) or acetaldehyde (CH₃CHO), produce nitrosamines from secondary amines in neutral and alkaline solutions of nitrite ion (Archer et al., 1976; Keefer and Roller, 1973). The reaction rates vary with steric accessibility to the nitrogen atom, but all are very much slower than those for nitrous acid in acidic solutions. The first mechanism proposed involved nucleophilic attack by nitrite ion on an iminium ion intermediate (R₂N⁺=CHR') followed by collapse of the adduct to nitrosamine. Equations 31, 32, and 33 show the formation of N-nitrosamines from nitrite ion, carbonyl compounds, and secondary amines.

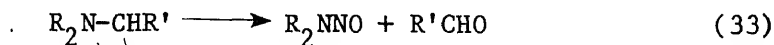


iminium ion





O=N-O





O=N-O

Alternative mechanisms have been discussed elsewhere (Keefer, 1979). This type of reaction may also explain the unexpected formation of nitrosamines from secondary amines and solid sodium nitrite (NaNO₂).

in cosmetics the bactericide 2-bromo-2-nitropropane-1,3-diol (HOCH₂CHBr(NO₂)CH₂OH; bronopol), which decomposes to release nitrite ion and formaldehyde (Schmeltz and Wenger, 1979).

Sodium nitrite has also been shown to produce nitrosamines from secondary amines at pH 11 in the presence of ferrocyanide ion [Fe^{II}(CN)₆]⁴⁻ (Maltz et al., 1971) and in 2,2'-bipyridine in the presence of cupric nitrate (Croisy et al., 1980). In both cases, interaction of nitrite ion with the metal salt is believed to generate a powerful nitrosating agent such as [Fe^{III}(CN)₅NO]²⁻ and [Cu^{II}(bipyr)(ONO₂), respectively. Many transition metals other than iron are known to form diverse nitrosyl complexes, but their ability to nitrosate amino compounds has not been extensively investigated.

Nitrosation by nitrate salts or nitric acid requires reductive conditions and, in principle, the formation of either nitrite ion, nitrogen dioxide, or nitric oxide intermediates. This is achieved in the presence of certain microorganisms. For example, the formation of nitrosamines from aqueous solutions of nitrate salts and secondary amines may be mediated by bacteria (Hashimoto et al., 1975; Hill and Hawksworth, 1972). Reduction of nitrate ion is difficult to achieve chemically. Recent work, however, shows that secondary amides are rapidly converted to their N-nitroso derivatives by nitric acid in acetic acid containing copper powder (McQuinn et al., 1979). Reduction of nitric to nitrous acid is believed to occur, effecting nitrosation heterolytically.

Other recent studies reveal that nitrosamines are readily formed when neutral aqueous solutions of sodium nitrate and secondary amines are exposed to either γ -irradiation (Challis et al., 1980) or to ultraviolet photolysis (Challis and Li, in press). The highest yields apply to experiments with excess sodium nitrate. These reactions are believed to result from reduction of nitrate ion to nitrogen dioxide, which then dimerizes to form the dinitrogen tetroxide reagent. This conclusion is supported by the concurrent formation of nitramines (Challis and Li, in press; Challis et al., 1980).

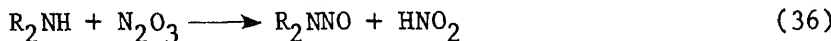
Nitrosation by Nitrogen Oxides

Nitrogen oxides are generated by the chemical and microbial reduction of nitrite and nitrate salts and are common environmental pollutants produced by combustion. Four of these compounds have been implicated in the formation of N-nitroso compounds: nitrogen dioxide, dinitrogen tetroxide, dinitrogen trioxide, and nitric oxide. The first three react unaided, but nitric oxide requires either oxidation to nitrogen dioxide or the presence of certain metal salts, iodine,

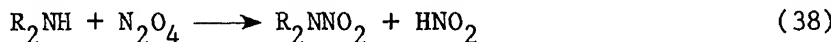
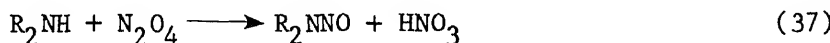
Gaseous dinitrogen trioxide and dinitrogen tetroxide exist in equilibrium with their nitric oxide and nitrogen dioxide constituents (Hisatsune, 1961):



Dissociation is less in condensed (liquid) than in gaseous phases (Grätzel et al., 1969, 1970; Redmond and Wayland, 1968; Shaw and Vosper, 1971). Dinitrogen trioxide reacts with amino compounds to give the N-nitroso product (Challis and Kyrtopoulos, 1979; Lovejoy and Vosper, 1968):



whereas dinitrogen tetroxide gives a mixture of N-nitroso and N-nitro compounds (Challis and Kyrtopoulos, 1978; White and Feldman, 1957):



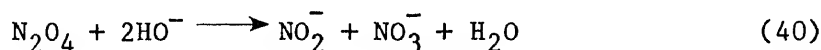
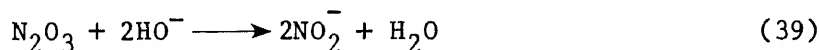
The extent of these reactions has not been widely recognized. They proceed in the gas phase, in organic (lipophilic) media, and in neutral and alkaline aqueous solutions.

Gas phase studies about smoking (Neurath et al., 1976; Spincer and Westcott, 1976) or atmospheric pollution (Bretschneider and Matz, 1973; Gehlert and Rolle, 1977; Hanst et al., 1977) show that both equimolar amounts of nitric oxide and nitrogen dioxide (i.e., dinitrogen trioxide) or nitrogen dioxide alone convert secondary amines to nitrosamines. Also, Pitts et al. (1978) and Atkinson et al. (1978) have detected both nitrosodiethylamine $[(\text{C}_2\text{H}_5)_2\text{NNO}]$ and nitrodiethylamine $[(\text{C}_2\text{H}_5)_2\text{NNO}_2]$ when either di- or triethylamine $[(\text{C}_2\text{H}_5)_2\text{NH}; (\text{C}_2\text{H}_5)_3\text{N}]$ reacts with low concentrations of nitric oxide and nitrogen dioxide under simulated atmospheric conditions. The possibility of such reactions in vivo is raised by the observation of nitrosomorpholine in the carcasses of mice (Iqbal et al.,

following inhalation of gaseous nitrogen dioxide. However, the nitrosomorpholine may form artifactually from a nitrosating agent produced in vivo from the nitrogen dioxide (Mirvish et al., in press).

In organic solvents, secondary amines react with gaseous dinitrogen trioxide to give high yields of N-nitrosamines (Challis and Kyrtopoulos, 1979; Lovejoy and Vosper, 1968), whereas gaseous dinitrogen tetroxide gives a mixture of N-nitroso and N-nitro compounds (Challis and Kyrtopoulos, 1978; White and Feldman, 1957). Furthermore, both dinitrogen trioxide and dinitrogen tetroxide have been recommended for the synthesis of N-nitrosamides in organic solvents (White, 1955). Both amines and amides are likely to react similarly in lipophilic media.

Nitrosation of amines by gaseous dinitrogen trioxide and dinitrogen tetroxide in aqueous solution is a recent discovery. Both would be expected to undergo rapid hydrolysis at pH > 5 to innocuous nitrite ion:



Hydrolysis does occur, but less rapidly than the nitrosation of many amines (Challis and Kyrtopoulos, 1978, 1979). Data in Table 4-3 show that substantial yields of nitrosamines are obtained within 4 minutes from secondary amines in neutral and alkaline solutions. With dinitrogen tetroxide, small amounts of nitramine form concurrently. Only the unprotonated amines react, similar nitrosamine ($\text{RR}'\text{NNO}_2$) yields are obtained for all but very basic amines ($\text{pK}_a < 1$), and, importantly, no reactions are observed with amides (Challis and Kyrtopoulos, 1978, 1979).

These results have been interpreted as evidence for tautomeric forms of dinitrogen trioxide ($\text{ONONO} \rightleftharpoons \text{ONNO}_2$) and dinitrogen tetroxide ($\text{ONONO}_2 \rightleftharpoons \text{O}_2\text{NNO}_2$), which appear to react with basic amines (Challis and Kyrtopoulos, 1978). Thus, nitrosation by gaseous dinitrogen trioxide and dinitrogen tetroxide follows the general mechanisms of equations 12 through 16, where NOY refers to ON-ONO or ON-ONO₂, respectively.

Subsequent work has shown that the reactions in aqueous media are inhibited by acid, sodium azide, ascorbic acid, some phenols

Nitrosation of Amino Compounds by Gaseous Dinitrogen Tetroxide and Dinitrogen Trioxide in Aqueous 0.1 M Sodium Hydroxide at 25°C^a

Amine	pK _a	Percent Nitrosation ^b	
		Dinitrogen tetroxide	Dinitrogen trioxide
Piperidine	11.12	39(0) ^c	65(0) ^c
Morpholine	8.33	19	52
N-Methylpiperazine	9.8, 5.11	33(44) ^c	39(45) ^c
Aniline	4.65	27	45
N-Methyl-4-nitroaniline	1.19	16	27
4-Nitroaniline	0.99	24(38) ^c	29(31) ^c
Diphenylamine	0.78	6	
3,5-Dinitroaniline	0.35	14	
2-Nitroaniline	-0.3	11	
2-Chloro-4-nitroaniline	-1.0	13	13
2,4-Dinitroaniline	-4.53	0	
N-Butylacetamide	-0.29	0	

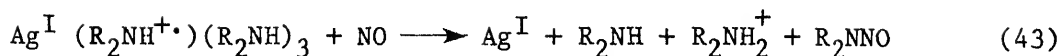
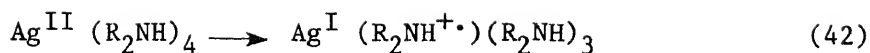
^aFrom Challis and Kyrtopoulos, 1979.

^bBased on [Amine].

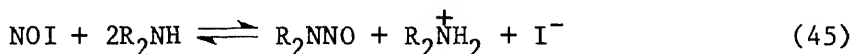
^cFigures in parentheses refer to reaction in phosphate buffer at pH 6.85.

however, by nucleophilic anions such as thiocyanate (Li, 1981) and by 1,2-alkanolamines and 1,2-dihydroxy compounds, including carbohydrates (Challis and Shuker, 1979, 1980; Challis *et al.*, 1980). Tertiary amines such as triethylamine react to give lower, but nonetheless significant, yields of nitrosamines (Li, 1981).

In the absence of catalysts, nitric oxide reacts slowly with secondary amines and amides under anaerobic conditions (Challis and Kyrtopoulos, 1978). These reactions can be accelerated by injecting air, which converts nitric oxide to nitrogen dioxide. Furthermore, under anaerobic conditions, the formation of nitrosamines from secondary amines and nitric oxide in aqueous ethanol is promoted by zinc iodide (ZnI₂), zinc bromide (ZnBr₂), cuprous chloride (CuCl), cupric chloride (CuCl₂), ferrous nitrate [Fe(NO₃)₂], silver nitrate (AgNO₃), silver perchlorate (AgClO₄), and other metal salts (Challis *et al.*, 1978). Many salts appear to generate amino radicals (R₂N•) or radical cations (R₂NH⁺•) which combine directly with nitric



Apart from oxygen or air, two of the best promoters for nitrosamine formation by nitric oxide are iodine (Challis and Outram, 1979) and hydrogen iodide (HI) (Outram, 1979). These, and zinc iodide, accelerate the reaction by forming nitrosyl iodide (NOI) (Outram, 1979):



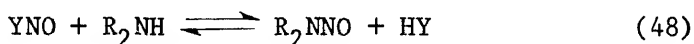
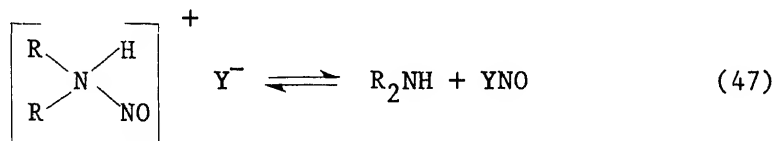
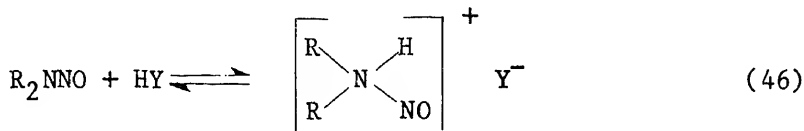
Transnitrosation: Nitrosation by Organic Nitroso and Nitro Compounds

In addition to nitrosation by nitrous acid and nitrogen oxides, certain nitroso compounds can act as nitrosating agents through the transfer of their nitroso group to an appropriate substrate (amines, amides, urea, amino acids). Depending on the substance that is nitrosated, these newly formed N-nitroso compounds may be carcinogenic. Nitrosation by nitrite esters (RONO), thionitrite esters [nitrosothiols (RSNO)], and certain other organic nitroso and nitro compounds has been known for many years, and some have been used in the synthesis of organic compounds. These compounds are formed by nitrosation pathways similar to those described for amino substrates.

Buglass et al. (1975) and, more recently, Singer et al. (1980) reported that transnitrosation is also effected by alicyclic nitrosamines, including certain derivatives of natural products such as nitrosoproline and nitrosopipericolic acid, and by piperazines, morpholines, and alkylpiperidines (Singer et al., 1980). These authors have reported that all of the nitrosopiperazines tested can be effective transnitrosating agents; the nitrosomorpholines are also active, but to a lesser degree. Nitrosopiperidine is stable and will not act as a nitroso donor, but various derivatives of nitrosopiperidine are transnitrosating agents. Nitrosoamino acids are also active but vary in their reactivity, depending on the basicity of the aminonitrogen (Singer et al., 1980).

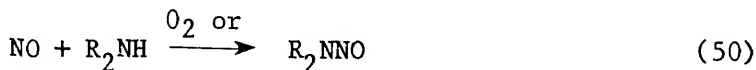
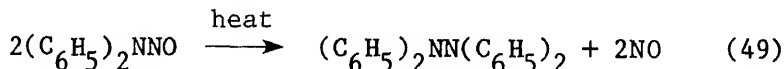
as nitrosating agents.

Transnitrosation by nitrosamines is facilitated when the N-N(0) bond is weakened by electron-withdrawing substituents (Challis and Osborne, 1973; Singer et al., 1980). Transnitrosation occurs in dilute acid (pH < 3) and is catalyzed by nucleophilic anions such as thiocyanate and iodide (Singer et al., 1980; Thompson and Williams, 1977). This implies that release of Y-NO is involved:



Thus, the scope and limitations of these reactions will be similar to nitrosation by nitrous acid. Since the acidic reaction conditions are not too dissimilar to those in the stomach, compounds such as nitrosodiphenylamine [(C₆H₅)₂NNO] and nitrosamino acids [RCH(N.NO)COOH] which are weak carcinogens or noncarcinogens, may produce strong carcinogens by reacting with amino compounds in vivo (Cardy et al., 1979).

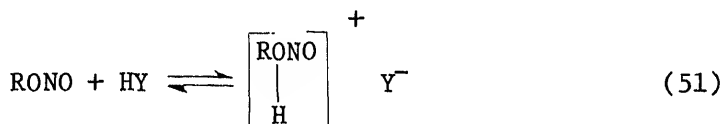
Transnitrosation can also be effected by heating aromatic N-nitrosamines to 50-80°C (Buglass et al., 1975; Rappe and Rydström, 1980). The ensuing nitric oxide requires oxidation to nitrogen dioxide (which will be in equilibrium with dinitrogen tetroxide) or catalysis by metal salts, etc., to react with another amine (Outram, 1979).



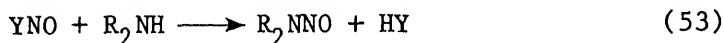
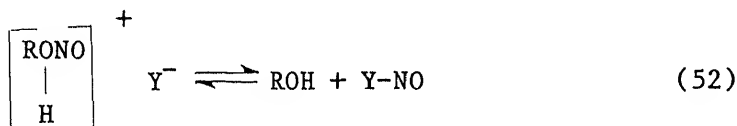
metal salt
catalysts

1976), nitrosoureas [$\text{H}_2\text{NCON}(\text{NO})\text{R}$] (Hallett et al., 1980; Singer, 1980), and nitrosourethanes [$\text{RO}_2\text{CN}(\text{NO})\text{R}'$] (Hallett et al., 1980) also release nitrous acid in dilute acid ($\text{pH} < 4$) and, in principle, are capable of transnitrosation to another amino substrate.

Nitrite esters derived from simple monohydric alcohols (e.g., $\text{C}_2\text{H}_5\text{ONO}$, $\text{C}_5\text{H}_{11}\text{ONO}$) behave as transnitrosating agents like nitrosamines. Thus, decomposition ensues in the presence of dilute acid ($\text{pH} < 4$) and nucleophilic anions to give Y-NO :

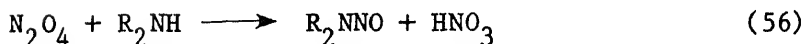


O-conjugate acid
intermediate

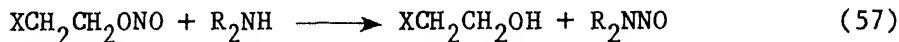


This can effect the nitrosation of amino compounds in the same way as for nitrous acid solutions (Aldred and Williams, 1981; Allen, 1954). Surprisingly, the O-conjugate acid intermediate does not seem to react directly with amino substrates (Aldred and Williams, 1981).

Decomposition of simple nitrite esters also proceeds thermally (Coombes, 1979) and photolytically (Forrest et al., 1978) to generate alkoxy radicals (RO^\bullet) and nitric oxide (NO). These reactions rarely occur under anaerobic conditions, and it is likely that ensuing nitrosation of amino compounds proceeds via dinitrogen tetroxide following oxidation of nitric oxide to nitrogen dioxide (equations 54 through 56). Since nitrite esters are very soluble in organic



Nitrite esters bearing electron-withdrawing β -substituents (X) are much more reactive, and they effect nitrosamine formation at ambient temperatures, in the absence of acid catalysts, by direct nucleophilic attack of the amine on the neutral nitrite ester (Challis et al., 1980; Challis and Shuker, 1979, 1980):



2-Ethoxyethyl nitrite ($\text{C}_2\text{H}_5\text{OCH}_2\text{CH}_2\text{ONO}$), for example, reacts with both piperidine [$(\text{CH}_2)_5\text{NH}$] and morpholine [$\text{O}(\text{CH}_2\text{CH}_2)_2\text{NH}$] in 0.1 M sodium hydroxide and with N-methylpiperazinium ion [$\text{HN}(\text{CH}_2\text{CH}_2)_2\text{NHCH}_3^+$] at pH 6.85 and 25°C to give significant yields of the corresponding N-nitrosamines in approximately 30 minutes (Challis and Shuker, 1979). Related reactions apply to nitrite esters derived from several vicinal diols [$\text{RCH}(\text{OH})\text{CHR}(\text{OH})$] (Challis et al., 1980), β -alkanolamines [$\text{RCH}(\text{OH})\text{CHRNHRR}'$] (Challis and Shuker, 1980), and carbohydrates (Challis et al., 1980). Table sugar (sucrose), milk sugar (lactose), and glucose, for example, form powerful nitrosating agents (presumably from the corresponding nitrite esters) on treatment with gaseous nitrogen dioxide, which rapidly converts secondary amines to their N-nitroso derivatives in aqueous alkali (Challis and Shuker, 1979; Challis et al., 1980). It is not known whether these reactions proceed in food or under mildly acidic conditions.

Certain nitrosophenols (e.g., nitrosocresols) and nitrosothiols (e.g., S-nitrosocysteine) can participate in transnitrosation reactions and, thus, can enhance nitrosamine formation. The mechanism whereby these reactions catalyze nitrosation by phenols and thiols is discussed earlier in the chapter (see "Catalysis").

Issenbart, 1970). Knowles et al. (1975) demonstrated that the interaction of liquid smoke emulsions and nitrite, in the presence of casein, produces nitroso- and nitrocresols. Although many nitrosophenols are unstable under aerobic conditions and are oxidized to the corresponding C-nitrophenol (nitrophenols do not nitrosate secondary amines), p-nitroso-o-cresol has been shown to catalyze the nitrosation of pyrrolidine by nitrite at pH 5 (Davies and McWheeny, 1977; Davies et al., 1978a).

Cysteine, a thiol, is an important amino acid residue in meat protein, and its concentration in meat has been reported to be 21-25 mM (Hamm and Hofman, 1966). Protein-bound nitrite is in the form of nitrosothiol groups (Olsman, 1977), and these cysteine derivatives are capable of nitrosating amines (Dennis et al., 1979); however, when bound to a peptide chain, their reactivity is greatly reduced (Massey et al., 1980).

Thionitrite esters (RSNO) should be more reactive than regular nitrite esters because RS^- is a more stable group than RO^- . This conclusion is partially substantiated by reports that alkyl- and arylthionitrites convert piperidine to its N-nitroso derivative in organic solvents (Oae et al., 1978) and that S-nitrosocysteine produces nitrosamines in acidic, neutral, and alkaline aqueous solutions (Davies et al., 1978a; Massey et al., 1980). Thus, there is clear evidence that activation by acids, which would generate free nitrous acid, is not necessary. Catalysis by air and by light (Oae et al., 1978) suggests that release of nitric oxide followed by oxidation to nitrogen dioxide (which will be in equilibrium with dinitrogen tetroxide) occurs in some cases.

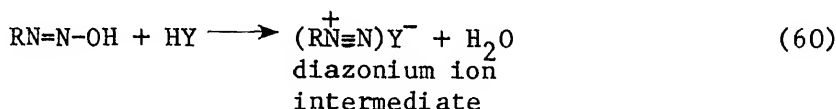
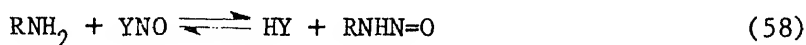
Early work reviewed by Fridman et al. (1971) showed that some aliphatic nitro compounds act as nitrosating as well as nitrating ($-NO_2$ donating) agents. More recently, the formation of nitroso-morpholine from tetranitromethane $[C(NO_2)_4]$, 2,2-dinitropropanol $[CH_3C(NO_2)_2CH_2OH]$, and 2-bromo-2-nitropropane-1,3-diol (bronopol) $[HOCH_2CBr(NO_2)CH_2OH]$ by heating with morpholine at 70°C has been described by Fan et al. (1978). The mechanism of these reactions is not clear, but one explanation is that release of nitrogen dioxide leads to formation of dinitrogen tetroxide, which then leads to nitrosation. It follows that any nitro compound that releases nitrogen dioxide may form nitrosamines from secondary and tertiary amines. Few other examples are known, but nitrodimethylamine transforms to nitrosodimethylamine upon heating or photolysis (Flournoy, 1962; Suryanarayanan and Bulusu, 1972). Furthermore, antianginal drugs containing a nitrate ester structure have been shown recently to produce nitrosamines in dilute acid, but these reactions are thought to proceed via the release of nitrous acid (Rasf

to effect the reaction. As discussed above, aromatic nitrosamines require a pH of 1-3 and the presence of a nucleophilic catalyst (e.g., thiocyanate) for the reaction to occur (Singer *et al.*, 1980; Thompson and Williams, 1977). Thus, such reactions could occur in the human stomach where the pH is sufficiently low and where nucleophilic catalysts are probably present. In contrast, nitrosothiols are present in meat products and are active at near neutral or alkaline pH's (Davies *et al.*, 1978a; Dennis, personal communication), and could be involved in transnitrosation reactions in the duodenum and small intestine. That such nitrosation reactions may occur *in vivo* is suggested by a study of Love *et al.* (1977) in which rats fed high doses of mononitrosopiperazine developed tumors that were similar to those that developed in rats fed lower doses of dinitrosopiperazine. However, despite the plausibility of these reactions taking place *in vivo*, knowledge about the occurrence of such reactions endogenously is extremely limited.

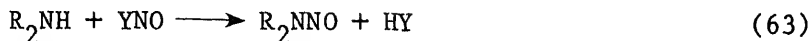
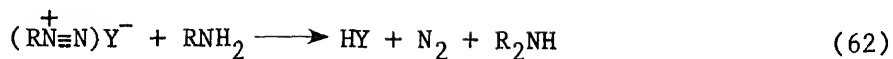
Nitrosation of Primary, Tertiary, and Quarternary Amino Compounds

At first sight, the formation of nitrosamines from these substrates seems unlikely, but there is good evidence to indicate otherwise. However, these reactions are usually less facile and/or less extensive than those with secondary amino compounds.

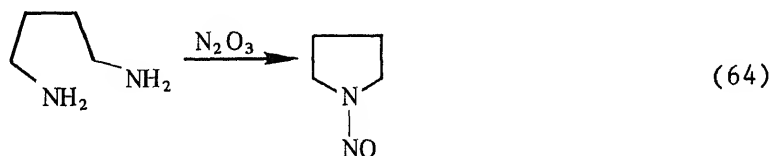
Nitrosation of primary aliphatic amines leads to deamination via an unstable diazonium ion intermediate, which reacts with nucleophiles to give substitution, elimination, and rearrangement products (Challis, 1981; Ridd, 1961):



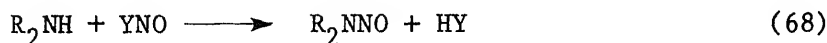
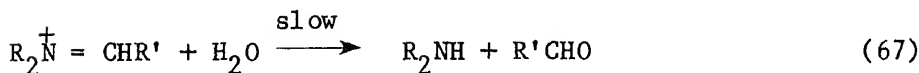
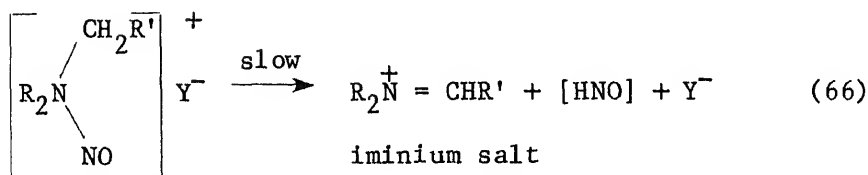
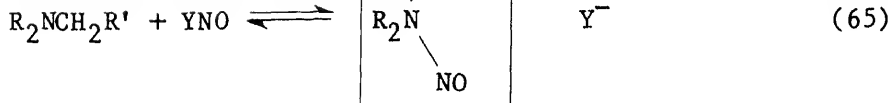
nitrosamine (Tannenbaum et al., 1978):



The kinetic characteristics of the initial deamination (including catalysis and inhibition) should be similar to nitrosamine formation, but yields of nitrosamines obtained from primary amines are very low because the diazonium ion intermediate reacts by several competitive pathways other than that shown in equations 62 and 63. Higher yields might be anticipated for reaction in organic (lipophilic) solvents, but this awaits confirmation. These reactions could be of some importance to the formation of heterocyclic nitrosamines from primary diamine precursors. Putrescine, for example, gives approximately 1.6% nitrosopyrrolidine on heating with nitrous acid (Warthesen et al., 1975):



Early work on the interaction of tertiary amines with acidified nitrite has been reviewed by Hein (1963) and by Smith and Loeppky (1967). Subsequently, these reactions attracted further attention (Gowenlock et al., 1979; Lijinsky and Singer, 1975) because of their role in the formation of nitrosamines, which is particularly facile for certain drugs such as aminopyrine (Lijinsky et al., 1972; Mirvish et al., 1974). The sequence of reactions presented in equations 65 through 68, in which an iminium salt undergoes hydrolysis to a secondary amine or reacts directly with nitrite ion to give a nitrosamine, was suggested by Smith and Loeppky (1967):



This scheme is supported by the recent identification of secondary amines (Singer, 1980) as coproducts with aldehydes, nitrous oxide (because $2HNO \rightarrow N_2O + H_2O$), and nitrosamines (Hecht *et al.*, 1978; Lijinsky and Singer, 1975). An alternative pathway involving electron transfer rather than nitrosation to form the iminium salt has been proposed by Michejda *et al.* (1976). This is a more likely mechanism with aromatic amines.

Temperatures between 50°C and 100°C are required for the nitrosation of tertiary alkylamines. Mirvish (1975) has estimated that these compounds are approximately 10,000 times less reactive than comparable secondary amines. This implies that either formation or hydrolysis of the iminium salt is rate-limiting (equations 65-68). Many investigators (e.g., Gowenlock *et al.*, 1979) have reported that the maximum rates of reaction with nitrous acid occur at pH 3-3.4 (similar to those of secondary amines), but there is considerable disagreement as to the dependence of kinetics on the concentration of nitrous acid (Gowenlock *et al.*, 1979; Ohshima and Kawabata, 1978; Singer, 1980).

Small amounts of nitrosodimethylamine have also been produced by the reactions of acidified nitrite with both quarternary methylammonium salts ($R_3NH_3^+Y^-$) and trimethylamine-N-oxide [$(CH_3)_3N^+ \rightarrow O^-$] (Eisenbrand *et al.*, 1975; Lijinsky and Singer, 1975; Ohshima and Kawabata, 1978). As for tertiary amines, these reactions require such conditions as high reagent concentrations and high temperatures.

(and in some cases nitrosamides) form when they react with acidified nitrite at elevated temperatures (Eisenbrand et al., 1975; Elespuru and Lijinsky, 1973; Lijinsky et al., 1972). Trialkylureas usually give the corresponding nitrosurea, whereas dialkyl- and trialkylthioureas, 1,1-dialkylureas, 1,1-dialkyl-3-phenylureas, and tetraalkylureas produce nitrosamines.

Factors Influencing Nitrosation in Foods

The extent to which nitrosation may occur in a food prior to ingestion is influenced by many factors. Nitrate or nitrite may be added deliberately for their preservative properties, or may be present naturally in water and some foods (see Chapter 5). Nitrate may be reduced to nitrite. Moreover, foods are exposed to nitrogen oxides that are either airborne contaminants or produced during certain processes such as the smoking, drying, or roasting of certain foods and baking in gas ovens. The extent of nitrosation in food is affected not only by the amount and type of nitrosatable compounds that are present, but also by the content of nitrosation catalysts and inhibitors and their solubility characteristics.

Smoking of Foods. Although wood-smoking is one of the oldest methods of food preservation, relatively little is known about the chemical interactions involved in this process. Most of the information pertains to the composition of the smoke and the components that affect the texture and flavor of the smoked products (Clifford et al. 1980; Gilbert and Knowles, 1975). According to Foster and Simpson (1961), wood smoke consists of two phases--a disperse, liquid phase containing smoke particles and a dispersing gaseous phase (vapor). Direct deposition of smoke particles on the food is believed to be negligible compared to the absorption of vapors by surface and interstitial water.

Smoking involves combustion, so it is highly probable that nitrogen oxides are absorbed by the food; however, the amounts absorbed have not been adequately measured. The nitrogen oxides may be expected to act as nitrosating agents (Challis and Kyrtopoulos, 1978, 1979; Challis et al., 1978) and, in principle, to produce N-nitroso compounds. It has been established that smoking produces up to a 55% reduction in the basic amino acid content, especially lysine (Clifford et al., 1980; Hoffman et al., 1977) and a smaller reduction of thio amino acids (Mauron, 1970). These losses could possibly be due to deamination by nitrogen oxides. In any event, the nitrogen oxides will undergo hydrolysis by surface and interstitial water, and nitrite and nitrate ions will be deposited in the food.

carbonyl compounds, esters (including isocyanates), aromatic amines, furans, and aromatic hydrocarbons (Gilbert and Knowles, 1975). The phenolic compounds, aromatic ethers, and furans react rapidly with nitrosating agents (Challis and Higgins, 1972; Challis and Lawson, 1971), and should therefore inhibit the formation of N-nitroso compounds (Challis, 1973). Inhibition by some of the phenolic constituents has been reported by Pignatelli et al. (1980), but the effect might be partly counteracted by the ability of nitrosophenols to catalyze the formation of nitrosamines (Davies and McWeeny, 1977; Walker et al., 1979). Certain carbonyl compounds may also catalyze the formation of nitrosamines from nitrite salts (Keefer and Roller, 1973).

SUMMARY

Nitrate and nitrite undergo a number of chemical and biological transformations. Of most importance to human health is their participation in both in vitro and in vivo nitrosation reactions, i.e., reactions that lead to the formation of N-nitroso compounds. There are factors that catalyze or inhibit these reactions in foods prior to consumption and in vivo.

Nitrate and Nitrite

Nitrate salts are stable, and they are not easily reduced chemically to nitrite salts. Nitrite salts, which are less stable, are readily oxidized to nitrate salts or reduced to nitric oxide, nitrous oxide, or nitrogen. Both nitrate and nitrite salts are transformed to reactive nitrogen oxides by gamma radiation and by photolysis.

Enzymatic reduction of nitrate salts and both enzymatic oxidation and enzymatic reduction of nitrite salts are well-known components of the nitrogen cycle. These transformations are readily accomplished in certain bacterial, fungal, and plant systems. They permit the interconversion of nitrate and nitrite salts and the generation of reactive nitrogen oxides from those salts.

Nitrosation

Amino substrates are nitrosated by electrophilic nitrosating agents derived from nitrous acid, dinitrogen trioxide, dinitrogen tetroxide, and, occasionally, nitric oxide.

nitrosating agent such as dinitrogen trioxide or the hydrated nitrosonium ion. Basic secondary amines react most rapidly at pH 3.4, but these reactions are generally slower than those for amides, ureas, and carbamates (e.g., urethane).

Nitrosation of secondary amines by aqueous nitrous acid is accelerated by nucleophilic anions such as thiocyanate and iodide and by some phenolic materials, thiols, and alkenes. Comparable reactions of amides, ureas, guanidines, and carbamates are not usually accelerated by these catalysts.

Nitrosation of secondary amines, amides, ureas, and guanidines by aqueous nitrous acid can be inhibited by compounds that reduce nitrous acid to nitrogen or nitric oxide. These inhibitors include ascorbic acid (vitamin C), α -tocopherol (vitamin E), and several naturally occurring polyphenolic antioxidants.

The nitroso group of certain nitrosamines, chiefly aromatic and alicyclic nitrosamines, can be transferred to amino substrates to form N-nitroso compounds. The catalysis of nitrosation by certain phenols and thiols, which proceeds via the formation of intermediate unstable nitroso compounds, may be termed transnitrosation. Transnitrosation has been demonstrated to occur in vitro, but little is known about its occurrence and, thus, its significance in nitrosation reactions in vivo.

Nitrosation of secondary amines by nitrite ion is exceptional, but it may occur slowly in aqueous solution in the presence of certain carbonyl compounds and in lipophilic solvents in the presence of metal salts. Nitrosamines form rapidly when aqueous solutions of secondary amines and either nitrite or nitrate salts are subjected to gamma radiation and to photolysis. After extended irradiation and photolysis, the nitrosamines are decomposed.

Nitrosamines form rapidly from reaction of secondary and tertiary amines with dinitrogen trioxide and dinitrogen tetroxide in organic and aqueous (neutral or alkaline) solutions. Nitric oxide forms nitrosamines only in the presence of oxygen, metal salts, or halide ions. These reactions are usually much faster than those occurring with aqueous nitrous acid, and they may explain the formation of nitrosamines by nitrogen oxide pollutants. Nitrosamine formation by dinitrogen trioxide and dinitrogen tetroxide is inhibited by reductants, but is catalyzed by common β -substituted alcohols such as alkanolamines, ethyleneglycol, and carbohydrates (sugars).

nitric oxide or nitrogen dioxide upon heating or nitrosonium ion upon treatment with acid, react with secondary amines to produce nitrosamines.

Under certain circumstances, primary, tertiary, and quarternary amino compounds (including amine oxides) produce nitrosamines upon reaction with nitrous acid and nitrogen oxides. Generally, these reactions are less extensive than those with secondary amines.

The extent of nitrosamine formation in foods is affected by the type of matrix (hydrophilic or hydrophobic), the presence of natural antioxidants, and methods of processing and cooking. For example, nitrogen oxides in smoke may produce nitrosamines in smoked products. These reactions may be mediated by other carbonyl and phenolic constituents of smoke.

CONCLUSIONS AND RECOMMENDATIONS

It is now clear that N-nitroso compounds form from nitrous acid and nitrite salts during the processing, cooking, and digestion of food. Thus, attention should be directed toward the conditions that prevail during the preparation, processing, and storage of foods to identify those that could lead to the generation of N-nitroso compounds. For example, the production of nitrite salts by the oxidation of amino compounds, the reduction of nitrate salts, and the hydrolysis of gaseous nitrogen oxides may all occur. Further research is also needed (1) to determine the rate at which N-nitroso compounds form at low nitrite concentrations, which approximate those present in the digestive tract; (2) to study the ability of dietary antioxidants other than ascorbic acid and α -tocopherol to inhibit the formation of N-nitroso compounds; and (3) to characterize the conditions that determine the formation of N-nitroso compounds in complex matrices that mimic food itself. It is also clear that N-nitroso compounds form readily from nitrogen oxides, but much more remains to be learned about the scope and extent of these reactions in the environment, during the cooking of food, and in vivo. Nitrogen oxides also generate N-nitro compounds from amino substrates, a reaction that warrants further attention in view of the carcinogenic properties of one of these compounds (dimethyl nitramine), which is the only one that has been tested.

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NITRATE, NITRITE, AND NITROGEN OXIDES: ENVIRONMENTAL
DISTRIBUTION AND EXPOSURE TO HUMANS

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NITRATE, NITRITE, AND NITROGEN OXIDES: ENVIRONMENTAL DISTRIBUTION AND EXPOSURE OF HUMANS

This chapter is a review of the environmental distribution, average concentrations, and ranges of concentrations of nitrate, nitrite, and nitrogen oxides. The data are used to estimate the exposure of humans to these substances. The accuracy of the estimates generated by the committee is limited by a number of weaknesses and uncertainties of current methods used to assay nitrate, nitrite, and nitrogen oxides. Moreover, in many cases, data on concentrations in the environmental sources surveyed are meager or outdated. The estimates of exposure from various foods are further limited by the possible inaccuracies in the data on average consumption. As more accurate and comprehensive data on environmental concentrations and consumption levels become available, the estimates developed by the committee can be refined and updated. In the meantime, the committee recommends that the estimates reported herein be used to determine the relative importance of various sources to the total exposure of the human population.

ANALYTICAL METHODS

A number of methods are used to analyze nitrate, nitrite, and nitrogen oxides. Each of them has limitations that should be considered when evaluating the data on the environmental concentrations.

Analysis of Nitrate

Methods for determining nitrate generally entail the use of spectrophotometry (Usher and Telling, 1975) based on the nitration of a phenolic compound, the oxidation of an organic compound by nitrate, or the reduction of nitrate to nitrite or ammonia. The most commonly used procedure is based on the reduction of nitrate to nitrite and subsequent colorimetric analysis of nitrite by the Griess reaction. (For a description of the Griess reaction, see the next section on analysis of nitrite.)

Several techniques have been developed to reduce nitrate. In the Follett and Ratcliff (1963) method, the substance under test is passed through spongy cadmium in a glass column with capillary inlet and outlet tubes. This technique results in essentially 100% reduction of nitrate to nitrite. For simultaneous determination of both the

developed by Elliott and Porter (1971) for meat extracts, in which nitrate is rapidly reduced by shaking the extract with spongy cadmium at a pH of 9.6 for 5 minutes. Other investigators have found that this technique results in a low recovery of nitrate, however, indicating inefficient reduction. Usher and Telling (1975) attributed the decreased reduction efficiency to the presence of polyphosphate in meat. This problem can be overcome by using a buffer with a buffering capacity twice as great as that used by Follett and Ratcliff (1963).

Usher and Telling (1975) have discussed several other general problems with the nitrate reduction technique -- dilution effect, interference from ascorbate, and interference from sulfur dioxide. These authors have concluded that, although this method is generally favored, it may not provide accurate determination of nitrate and nitrite at levels below 10 mg/kg.

Several investigators have described methods in which ion-selective electrodes are used to measure nitrate in extracts of various foods, including spinach (Barker et al., 1971; Voogst, 1969), sugar beets (McCaslin et al., 1970), and baby foods (Liedtke and Meloan, 1976; Pfeiffer and Smith, 1975). Unfortunately, since the nitrate electrode is nonspecific, other ions interfere in the analysis, and steps must be taken to compensate for, or eliminate, their influence (Usher and Telling, 1975). For example, chloride can interfere in nitrate measurements when it is present in quantities 10 or more times greater than the quantity of the nitrate, a situation that exists in cured meats and certain cheeses (Comer, 1978). But there are several methods for removing the chloride ion. For example, the silver resin method proposed by Paul and Carlson (1968) eliminates chloride interference in the analysis of baby foods, including fruits, vegetables, formulated foods, and meats. Pfeiffer and Smith (1975) compared the nitrate electrode method with the xylenol method of the Association of Official Analytical Chemists (AOAC). They reported that the standard error when measuring nitrate at 100 mg/kg was 4.3 mg/kg.

A rapid method for the simultaneous determination of nitrate and nitrite has been developed using high-pressure ion-exchange liquid chromatography (HPLC) to separate the ions and ultraviolet absorption at 210 nm for their detection (Gerritse, 1979; Thayer and Huffaker, 1980). The method can determine as little as 50 pmol of nitrate in samples ranging from 5 to 500 μ l, and has recently been applied to food samples (Leuenberger et al., 1980).

1973). In the Greiss reaction, which was adopted by the AOAC as the official method for assaying for nitrite (Horwitz, 1975), sulfanilamide is nitrosated to form an azo compound, which is coupled with 1-naphthylamine to yield a pink dye.

In a modification of the official AOAC method, nitrite is detected by the diazotization of sulfanilamide and subsequent formation of the azo dye after coupling with N-(1-naphthyl)ethylenediamine (Fiddler, 1977). However, since the coupling reagent is a primary amine, it can react with nitrite under the acidic conditions present during analysis and compete with sulfanilamide for the available nitrite. Therefore, if the sulfanilamide and the coupling agent are added together, this competing reaction can lead to the destruction of some nitrite, loss of color, and inaccurate determinations of nitrite (Sen and McPherson, 1978). For this reason, the modified official AOAC method calls for the separate addition of the two color reagents (Fiddler, 1977). Sulfanilamide is added first, and after 5 minutes (when the diazotization reaction is complete), N-(1-naphthyl)ethylenediamine is added to form the pink azo dye. Sen and McPherson (1978) have demonstrated that ascorbate present in food samples can destroy some of the nitrite during preincubation with sulfanilamide. They have suggested an alternative method of analysis in which ascorbic acid, in the amounts normally found in foods and cured meat products, does not interfere. Another problem with this method is that breakdown products of other chemicals may be included in the measurements of nitrite. For example, Pyper and Hartman (personal communication) found that oxyhyponitrite behaves as a mixture of nitrate and nitrite when assayed by the modified AOAC procedure.

Several new procedures have greatly increased the sensitivity of determinations for nitrite in foods and other substances. Walters et al. (1978) acidified food samples with acetic acid and then measured the resulting nitric oxide with a chemiluminescence detector. Although this method does have the potential of being at least one order of magnitude more sensitive than the colorimetric methods, it has certain limitations (Doerr et al., 1981). For example, water was found to decrease the response of the method, thereby limiting its applicability. Furthermore, only acidification of the sample is used to produce nitric oxide from nitrite. However, nitrous acid decomposes by mono-, bi-, and trimolecular reactions to form a number of N-nitrosating species, including the nitrosonium ion (NO^+), as well as the nonnitrosating nitric oxide (Doerr et al., 1981). The amount of nitric oxide that can be produced is dependent upon a balance among competing reactions that can be altered by various compounds present in foods. For example, the presence of nitrosat-

for measuring nitrite is more than 600 times more sensitive than the colorimetric procedure.

Doerr et al. (1981) have also assessed the effectiveness of the chemiluminescence procedure for determining nitrite and compared it with the Griess colorimetric method. The test medium was a meat slurry containing either ascorbate or cysteine, both of which can cause loss of nitrite in meat (Fox and Nicholas, 1974) or interfere in the analysis. To facilitate a comparison of the effectiveness of these analytical procedures when they are free of interfering substances, the investigators added charcoal to the test system to eliminate the ascorbate interference. In both methods sodium nitrite values were comparable in samples without an added reductant, regardless of whether they had been treated with charcoal or not. Unlike the Griess method, the addition of a reductant in the chemiluminescence method did not reduce the amount of nitrite measured. The nitrite values obtained with the two methods were equivalent after treatment with charcoal.

A new method for determining nitrite by HPLC (Gerritse, 1979; Thayer and Huffaker, 1980) was discussed above in the section on nitrate. Concentrations of nitrite as low as 200 pmol can be detected in samples ranging from 5 to 500 μ l.

Special difficulties are encountered when measuring nitrite levels in meat because nitrite combines with some constituents and may not be measured in the assays just described (Cassens et al., 1974). Thus, for each of the techniques described, it is important to know which nitrite species are measured when meat samples are being analyzed. Currently, it is not clear whether all nitrite present in meat is actually measured. However, it appears that similar species may be measured by the colorimetric and chemiluminescence methods since results from these methods are comparable.

Analysis of Nitrogen Oxides

Because of the complex chemistry of the nitrogen oxides, any of the following gases may exist in ambient air: nitrous oxide, nitric oxide, nitrogen dioxide, nitric acid, and nitrous acid. Both nitric oxide and nitrogen dioxide are stable free radicals, and they typically participate in rapid simultaneous reactions. For example, they may combine to form dinitrogen trioxide and dinitrogen tetroxide. It has been extremely difficult to prepare accurately known concentrations of nitrogen dioxide because of its high reactivity with impurities, other pollutants, and the walls of the system. This section briefly reviews common manual and

based on the colorimetric Griess reaction described previously. In this method, nitrogen dioxide is reacted with diazotizing-coupling reagents to form a deeply colored azo dye. Prior to the establishment of air quality standards, this method was adopted by the Inter-society Committee (1972). The original U.S. Environmental Protection Agency (EPA) reference method for nitrogen dioxide, which was published along with the air quality standard (U.S. Environmental Protection Agency, 1971b), was based on the adaptation of the Griess-Saltzman method to a 24-hour integrated manual method (Hochheiser, 1965; Jacobs and Hochheiser, 1958). In 1972, the EPA published a notice in the Federal Register indicating that this modified method was unreliable (U.S. Environmental Protection Agency, 1972). As a result, an extensive effort was undertaken to develop and validate more reliable procedures.

After extensive interlaboratory testing, the EPA finally selected chemiluminescence as the reference method for nitric oxide and nitrogen dioxide, but it listed the sodium arsenite and TGS-ANSA (triethanolamine-guaiacol-sulfite/8-amino-1-naphthalenesulfonic acid-ammonium salt) methods as being equally reliable (U.S. Environmental Protection Agency, 1976a,b).

Chemiluminescence Method. Atmospheric nitrogen dioxide is determined indirectly by photometric measurements of the light intensity at wavelengths greater than 600 nm, which result from the chemiluminescent reaction of nitric oxide with ozone. Nitrogen dioxide is first quantitatively reduced to nitric oxide by a thermal converter. Since nitric oxide, which commonly exists in ambient air with nitrogen dioxide, passes through the converter unchanged, the resultant total NO_x concentration is equal to nitric oxide plus nitrogen dioxide. A sample of input air is also measured without having passed through the converter. The latter measurement (background nitric oxide) is then subtracted from the measurement of total NO_x to determine the concentration of nitrogen dioxide.

This method requires dynamic calibration with known quantities of nitric oxide and nitrogen dioxide. Nitric oxide samples of known concentrations, which are stored in high-pressure cylinders to ensure stability, are commercially available, whereas nitrogen dioxide samples of known concentrations can be generated either by a gas phase titration (GPT) technique or by nitrogen dioxide permeation devices.

Sodium Arsenite Method. The sodium arsenite method is a 24-hour integrated manual method similar to the original EPA reference method (Christie et al., 1970). Nitrogen dioxide is collected by bubbling

form an azo dye, which is then measured colorimetrically.

TGS-ANSA Method. The TGS-ANSA method is also a 24-hour integral manual method. Nitrogen dioxide is collected by bubbling air through an orifice-type bubbler into a solution of triethanolamine, o-methoxyphenol (guaiacol), and sodium metabisulfite. The nitrite ion produced during sampling is determined colorimetrically by reacting the exposed absorbing reagent with sulfanilamide and 8-amino-1-naphthalenesulfonic acid-ammonium salt (ANSA).

Summary

Classical methods for the determination of nitrate are generally unreliable since their success is based on the degree to which nitrate is reduced to nitrite -- a reaction that is difficult to control and reproduce. Other techniques to measure nitrate, such as the ion-selective electrode techniques, are subject to many interferences, which necessitate further clean-up steps.

The successful determination of nitrite is not as difficult as it is for nitrate. Once in solution, free nitrite is readily determined by diazotization and coupling reactions or, more recently, by chemiluminescence techniques, which are 600 times more sensitive than the colorimetric procedures. In addition, the colorimetric methods give incorrect, lower nitrite values than the chemiluminescence methods in the presence of reductants such as ascorbate. However, the addition of charcoal removes these reductants, whereupon the nitrite values measured by the colorimetric methods are equivalent to those measured by chemiluminescence techniques.

Based on its accuracy and freedom from many known interferences the chemiluminescence method is recommended as a reference technique not only for cured meats but also for other samples of biological origin; however, the high cost of the required instrumentation (e.g. a thermal energy analyzer) will probably preclude its wide acceptance as a routine method for determination of nitrite.

In the following section on environmental distribution, the values used for nitrate and nitrite have been taken from the published literature. A variety of test methods were used to arrive at these data, and not all investigators described the recovery from control samples to which varying concentrations of nitrate or nitrite were added to measure any oxidation or reduction that may have occurred in the sample prior to testing. Even when the procedures are properly controlled, incorrect readings can result from the presence of reducing agents (e.g., when reductants are present during the use

ENVIRONMENTAL DISTRIBUTION

Major sources of nitrate and nitrite intake are food and water. Nitrogen oxides are found primarily in polluted air (in the ambient atmosphere, in indoor environments, and in the workplace) and in tobacco smoke. In this section, data on the amounts of nitrate, nitrite, and nitrogen oxides in various environmental sources are reviewed. Despite the limitations of the various methods for measuring these chemicals (especially nitrate and nitrite) and the limited amount of data available on their concentrations in the environment, the committee has assumed that published measurements are a reasonably accurate reflection of the relative levels present in environmental media to which the human population is exposed.

Food

The committee recognizes that the concentrations of nitrate and nitrite vary considerably within each food category. Nonetheless, it has estimated average concentrations and has used these estimates later in the chapter to project the average intake of these ions from food. These estimates have been developed to compare the relative contribution of various foods to the total intake of nitrate and nitrite and to compare exposure from these sources with exposure from other environmental media, such as water and air.

Considerable confusion has occurred in the past when the nitrate and nitrite contents of various foods were compared on the basis of published data that were expressed in several different molecular forms (Phillips, 1968b). For example, the nitrate or nitrite content of foods is commonly expressed in terms of the sodium salt, the nitrate or nitrite ion, or as nitrate- or nitrite-nitrogen. In this chapter, the concentrations of nitrate and nitrite are expressed in terms of the two ions (NO_3^- and NO_2^-). If the data taken from the literature were expressed in different terms, appropriate conversions were made.

In many cases, the committee relied on the same data base as that used by White (1975, 1976) to estimate relative exposure to dietary sources of nitrate and nitrite. When data from other comparable surveys were available, the committee used them as a supplement to this data base in an attempt to refine and update the figures used by White.

Me Products: Nitrate and Nitrite. Selected data

NOTE: Many of these values have been converted from concentrations of the salt to concentrations of the ion, and some have been rounded off to significant figures.

Product	No. of Samples Tested ^a	Nitrate Content, mg/kg		Nitrite Content, mg/kg		Year	Reference
		Range	Average	Range	Average		
Bacon, uncooked (nitrate-cured) ^b	12			37-430	140	1926	Kerr <i>et al.</i> , 1926
Bacon, uncooked (nitrite-cured)	12			8-63	28	1972	Sen <i>et al.</i> , 1974
	14			7-68	35	1974	Sen <i>et al.</i> , 1975
	12	ND ^c -64	33	ND-88	42	1975	Sen <i>et al.</i> , 1977
	20	7-320	96	3-170	25	1972	Panalaks <i>et al.</i> , 1973
	12			4-32	17	1974	Greenberg, 1977
	316 ^d				40 ^d	1977	American Meat Institute, 1977
	82 ^e				21 ^e	1978-79	American Meat Institute, personal communication
	2,476 ^{e,f}				12 ^{e,f}	1978-79	M. Nelson, U.S. Department of Agriculture, personal communi- cation, 1981
	14			7-68	35	1976	Greenberg, 1977
Bacon, fried	12	4-98	32	5-18	7	1973	Panalaks <i>et al.</i> , 1974
Bologna	21	9-220	87	<0.7-150	29	1972	Panalaks <i>et al.</i> , 1973
	3	26-60	42	<0.7-7	5	1973	Panalaks <i>et al.</i> , 1974
	20			3-55 ^g	31 ^g	1977-78	Buege <i>et al.</i> , 1978
Ham (nitrate- cured) ^b	12			24-640	280	1926	Kerr <i>et al.</i> , 1926
Ham (nitrite- cured)	23	0.7-1,400	150	<0.7-140	29	1972	Panalaks <i>et al.</i> , 1973
	19				16	1979-80	Birdsall, 1981
Ham, fried	20			4-220	37	1974	Greenberg, 1977
Ham, pasteurized, canned, cured	1	140	140	99	99	1972	Panalaks <i>et al.</i> , 1973
Calami, European- type sausages	25	4-270	89	<0.7-66	17	1972	Panalaks <i>et al.</i> , 1973
	63	4-540	78	5-97	13	1973	Panalaks <i>et al.</i> , 1974
Sausages, fermented	25				6 ^g	1977	American Meat Institute, 1977
Sausages, smoked and unsmoked (nitrate-cured) ^g	13			13-940	190	1926	Kerr <i>et al.</i> , 1926
Sausages, smoked and unsmoked (wieners and sausages)	18	17-240	110	<0.7-52	9.6	1972	Panalaks <i>et al.</i> , 1973
Sausages, smoked and unsmoked (wieners)	2	66, 130	96	10, 10	10	1973	Panalaks <i>et al.</i> , 1974
Sausages, smoked and unsmoked	10			0-50	31	1975	Coppola <i>et al.</i> , 1976
	20			5-43 ^g	24 ^g	1977-78	Buege <i>et al.</i> , 1978
	152				19 ^g	1979-80	Birdsall, 1981
Sausages, pasteurized, canned, cured	2	15, 18	16	5, 7	6	1973	Panalaks <i>et al.</i> , 1974
Sausage, summer shelf-stable canned cured meat	20			0-29 ^g	6 ^g	1977-78	Buege <i>et al.</i> , 1978
	16	<0.7-840	100	<0.7-17	6	1972	Panalaks <i>et al.</i> , 1973
	7	<0.7-110	26	5-8	6	1973	Panalaks <i>et al.</i> , 1974
	3,944				19 ^g	1977	American Meat Institute, 1977
Refrigerated canned meats	242				44 ^g	1977	American Meat Insti- tute, 1977

^aConcentrations, expressed as the nitrate and nitrite ion, were measured in products at the retail level, unless otherwise designated.

^bRepresentative of nitrite concentrations found after curing in nitrate pickle, in common practice before USDA regulations became effective in 1926 (U.S. Department of Agriculture, 1926). In earlier times, however, many meat products were dry-cured and aged at ambient temperatures without any nitrate or nitrite additions (Kemp *et al.*, 1974, 1975).

products during the curing process. All data were taken from literature published during the past decade except for the data pertaining to "nitrate-cured" bacon, sausages, and ham, which illustrate the high nitrite content of cured meats in the United States during the mid-1920's. The large decline in the average nitrite content of these products since that time reflects both the impact of federal regulations and the changes engendered by the meat-packing industry.

One of the most striking aspects of the data presented in Table 5-1 is the wide range of nitrate and nitrite concentrations found within certain product categories. In some cases, average concentrations have been considerably influenced by only a few measurements of high concentrations. Data from many of the surveys cited in this table indicate that a significant percentage of the products tested contained low levels of residual nitrite. For example, among the 297 products analyzed by Panalaks et al. (1973, 1974), 193 (65%) contained residual nitrite at approximately 10 mg/kg or less.

In addition, 127 products (43%) contained nitrite at levels of 7 mg/kg or less. Thus, nearly one-half of the Canadian products surveyed by Panalaks in 1972 and 1973 contained the same level as the average in cured meat products from Norway (Table 5-2), where the total amount of nitrate and nitrite used as food additives has decreased by more than 80% since 1973 (Ringén, personal communication, 1981).

White (1975, 1976) estimated the intake of nitrate from cured meats for the U. S. population. His estimate was based on a survey by Fudge and Truman (1973), who reported levels of nitrate in 171 samples of predominantly European meat products. The average concentration of nitrate in these samples was approximately 200 mg/kg (Table 5-2). Since there has been no comparable survey of U. S. products, the committee has used White's data as a basis for estimating the average concentration of nitrate in cured meats. These data were modified to reflect some changes made since White's survey. For example, a ban on the addition of nitrate to all but a few products was enacted in Canada (Health and Welfare Canada, 1975). As a result, there has been a fivefold decrease in the nitrate content of Canadian products between 1971 and 1978 (Table 5-2). The trend toward decreasing residual nitrate concentrations in cured meats can be discerned by comparing the average concentrations measured in several surveys of Canadian products conducted over the last decade. Although similar regulations have not been adopted in the United States, the meat industry has taken internal action to reduce the nitrate content of cured meats. However, there has been no close monitoring of such concentrations to determine how effective this voluntary program has been. In order to develop a basis for U. S. products, the

Average Residual Nitrate and Nitrite Concentrations in Cured Meats^a

NOTE: Some of these values have been converted from concentrations of the sodium salt to concentrations of the ion, and some have been rounded off to two significant figures.

<u>Source of Data</u>	<u>Nitrate, mg/kg</u>	<u>Nitrite, mg/kg</u>	<u>References</u>
1971 Survey of Canadian products ^b	130	19	Panalaks <u>et al.</u> , 1973
1973 Survey of Canadian products ^b	64	11	Panalaks <u>et al.</u> , 1974
1978 Survey of Canadian products ^b	28	15	C. J. Randall, personal communication, 1979
1936 Survey of U.S. products ^b	NR ^c	30	Kolari and Aunan, 1972
1937 Survey of U.S. products ^b	NR	42	Kolari and Aunan, 1972
1971 Survey of U.S. products ^d	NR	23	Kolari and Aunan, 1972
1972 Survey of U.S. products ^e	NR	32	Kolari and Aunan, 1972
1978 Survey of U.S. products ^b	28	11	C. J. Randall, personal communication, 1979
1972 Survey of European Products	210	14	Fudge and Truman, 1973
1976 Survey of Norwegian products	25	8	Lyng, 1978

^aThese averages do not take consumption patterns into consideration.

^bProducts examined at the retail level.

^cNR = no data reported.

^dProducts examined within 1 to 14 days after production.

^eProducts examined within 1 to 2 days after production.

than the only value for the nitrate content of U.S. products identified by the committee (Randall, personal communication, 1979).

In contrast to the clear trend of a decrease in residual nitrate in cured meats seen in the Canadian data, no distinct pattern has been observed for residual nitrite (Table 5-2). In 1978, an apparent decrease in residual nitrite was detected in bacon (American Meat Institute, personal communication). The significance of the observed drop, however, is unclear because ascorbate is known to interfere with the detection of nitrite, and, since 1978, bacon samples have contained substantial amounts of ascorbate and isoascorbate. Moreover if the nitrite was not measured immediately after processing, the low levels could be attributed to decreases in residual nitrite that occur over time after processing. However, these explanations are merely conjectural since the details of this study have not yet been published. In contrast, some investigators have reported an increase in the nitrite content of some cured meat products (Sen et al., 1977; U.S. Department of Agriculture, 1976); however, these findings were not statistically significant. Moreover, differences detected in the study by the U. S. Department of Agriculture (USDA) may have resulted from sampling the products at different time intervals after processing (U.S. Department of Agriculture, 1978a).

The decrease in detectable nitrite levels in cured meats after processing is due primarily to its reactivity (Cassens et al., 1979). Because of this reactivity with various components in meat, less than 50% of the nitrite added can be analyzed chemically shortly after processing (Cassens et al., 1974). Before 1974, little was known about the fate of added nitrite, except for the cured meat pigment (nitric oxide myoglobin) and the residual nitrite. Although Pivnik et al. (1967), Nordin (1969), and Herring (1973) demonstrated rapid decreases in nitrite in model systems or in commercially processed meat products, these investigators did not account for the nitrite lost. Since then, Fujimaki et al. (1975), Sebranek (1974), and Cassens et al. (1977) have demonstrated that nitrite combines with both the water- and salt-soluble meat fractions. Although much of the protein-bound nitrite exists as S-nitrosothiols, which can participate in nitrosation reactions (Davies et al., 1978), Massey et al. (1980) have reported that the rate of nitrosation by S-nitrosocysteine is considerably reduced when the nitrosocysteine is incorporated into simulated peptide chains. Thus, the importance of bound nitrite in nitrosation reactions remains unclear.

The committee's estimates of the nitrite content of cured meats and subsequent exposure of humans from this source (see next section) rely exclusively on data concerning residual nitrite, and, because it is not clear whether bound nitrite is measured in current assays

<u>Source</u>	<u>Nitrate</u>	<u>Nitrite</u>	<u>Nitrogen Oxides</u>
Cured meats	40 mg/kg	10 mg/kg	
Fresh meats	10 mg/kg	1 mg/kg	
Vegetables	NA ^a	NA ^a	
Fruits	20 mg/kg	0 ^b	
Baked goods and cereals	12 mg/kg	2.6 mg/kg	
Milk and milk products	0.5 mg/liter	0 ^b	
Water	1.3 mg/liter	0 ^b	
Ambient atmosphere			0.058 mg/m ³
Tobacco smoke			0.51 mg/cigarette

^aNot applicable. Overall average concentrations in vegetables were not used in exposure calculations. See Table 5-8 for individual averages for 35 vegetables.

^bNitrite concentration was considered to be negligible.

In 1975, White estimated the nitrite content of cured meats by using the broad data base developed by Kolari and Aunan (1972), which included data collected from more than 950 samples of meat products tested at the retail level in 1936 and 1937 and 1 to 14 days after processing in 1971 and 1972 (Table 5-2). The committee has used the average nitrite value for the most recent year of this survey -- 32 mg/kg in 1972 -- to estimate the average residual nitrite in cured meat products. However, because nitrite was measured shortly after processing, and the committee wished to develop an estimate indicative of the nitrite content at the time of consumption, it has reduced this estimate based on average decreases in residual nitrite occurring between processing and consumption.

Birdsall (1981) estimated the average nitrite content of cured meats at the time of consumption to be approximately 7 mg/kg. This is approximately a 70% decrease from the 24 mg/kg reported as the average amount detected in products tested shortly after processing (American Meat Institute, 1977). The lower figure (7 mg/kg) is the average of measurements taken at 15 to 28 days after processing for noncanned items, at 29 or more days after processing for refrigerated

TABLE 5-4

Average Residual Nitrite (mg/kg) in Cured Meats at Specified Times After Packaging when Stored at 4.4°C to 7.2°C^a

NOTE: Concentrations of sodium nitrite used in the original estimates have been converted to nitrite ion.

Category	Nitrite Concentration, mg/kg (and Number of Samples) at Different Stages (No. of Days) after Packaging				
	≤ 3 Days	4-6 Days	7-14 Days	15-28 Days	29-59 Days
Cured sausage	40 (N = 316)	13 (N = 45)	12 (N = 19)	9 (N = 116)	6 (N = 31)
	6 (N = 25)	7 (N = 5)	7 (N = 8)	6 (N = 40)	6 (N = 27)
	9 (N = 32)	9 (N = 17)	15 (N = 18)	8 (N = 42)	6 (N = 25)
	27 (N = 604)	13 (N = 60)	13 (N = 112)	7 (N = 91)	5 (N = 113)
Cured meats, generated	44 (N = 242)	40 (N = 98)	34 (N = 8)	15 (N = 22)	9 (N = 64)
Cured meats, stable ^b	[-----19-----]				
	[------(N = 3,944)-----]				

^a Birdsall, 1981.
^b at ambient temperatures.

products at the time of processing and consumption reported by Buege et al. (1978) and the 85-98% loss reported by Cassens et al. (1979).

If a reduction factor of 70% is assumed for Kolari and Aunan's 1972 average of 32 mg/kg, the residual nitrite concentration in products at the consumer level would be 10 mg/kg. For the purpose of estimating the residual nitrite content of cured meats at the time of consumption, the committee has used this amount -- 10 mg/kg (Table 5-3).

Fresh Meat Products: Nitrate and Nitrite. Fresh and unprocessed meat products, which constitute more than half of the meat sold in the United States, are generally not regarded as major contributors to the nitrate and nitrite ingested by humans. For example, White (1975) did not include fresh meat in his estimates of dietary sources of these ions because "no definitive information on the amount of nitrate or nitrite in fresh meat was located."

The committee has reviewed several studies of the nitrate content of fresh meat products. In one of these, Kačmár and Bartík (1965) found nitrate concentrations of approximately 170 mg/kg in fresh pork muscle; however, most other investigators who have studied nitrate in the fresh muscle of various animal species have reported much lower concentrations. For example, Whelan (1935a,b) found nitrate concentrations of approximately 6 mg/kg and 33 mg/kg in the tissues of dogs fed diets containing moderate and high concentrations of nitrate, respectively.

Wright and Davison (1964) reported nitrate concentrations of 0.9 mg/kg in the meat of dairy cows used as controls in a sodium nitrate feeding study and 11 mg/kg in the meat of cattle fed hay supplemented with close to lethal levels of sodium nitrate. Usher and Telling (1975) reviewed the data for meat blanks (controls without added nitrate or nitrite) given in a number of independent studies and found that the reported nitrate concentrations ranged from 0 to 49 mg/kg. They estimated that the level of sensitivity of the assay method(s) used in the studies was 6 mg/kg. Skovgaard (1980) reported a nitrate concentration of 19 mg/kg in bacon to which no nitrate or nitrite had been added, and Christiansen et al. (1973) found 55 mg/kg in comminuted hams after cooking.

There is little information on the nitrite content of fresh meats, but there are data indicating that free natural nitrite levels in meats processed without added nitrite are generally low. In 1979, approximately 3 billion kilograms of such products was processed without added nitrite (American Meat Institute, 1980; U.S. Department of Agriculture, 1980). Empata (1951) reported that dry-cured

the nitrite. Christiansen et al. (1973) found concentrations of nitrite ranging from 1 to 8 mg/kg in ground fresh hams stored from 7 to 168 days at 7°C.

To develop an estimate of human intake from this source, the committee has assumed that meat products to which no nitrate or nitrite has been added contain nitrate concentrations of 10 mg/kg and that the nitrite content is only sporadic, averaging 1 mg/kg (Table 5-3).

Vegetables: Nitrate and Nitrite. Nitrate concentrations of vegetables are listed in Tables 5-5A and 5-5B. The tremendously wide ranges in the nitrate levels of certain vegetables are not due merely to diversity among extraction and other assay procedures. To a great extent, they reflect true variations in the nitrate content of different samples of the same type of vegetable. It is important to recognize that information on the nitrate content of vegetables without information on the content of various nitrosation inhibitors, especially ascorbic acid, may be misleading. Thus, later in this section, average concentrations of nitrate and ascorbate in various vegetables are compared.

During growth, the nitrate content of vegetables is affected most by nitrogen supply and light conditions (Corré and Breimer, 1979), although a number of factors may be involved (Corré and Breimer, 1979; Maynard, 1978; Maynard et al., 1976).

For example:

- Related plant strains (cultivars) systematically differ in nitrate content.
- Different levels and timing of nitrogen fertilizer application affect the nitrate content. Generally speaking, nitrate accumulation increases as the amount of nitrogen fertilizer used increases and if the fertilizer is applied shortly before harvest.
- Nitrate levels tend to increase as daytime temperatures drop below an optimal temperature. Thus, geographic region and season of harvest affect nitrate content.
- Greenhouse plants tend to accumulate higher levels of nitrate than do plants grown outdoors, perhaps because nitrogen fertilizers are used more heavily indoors.
- Plants grown in shade, at high latitudes with limited sun-

Average Concentration of Nitrate, mg/kg Fresh Weight,
Except for Wilson, 1949^a

Except for Wilson, 1949a							
Vegetable	Fresh or Canned (individual studies)				Frozen (individual studies)		
	Richardson, 1907	Wilson, 1949a	Jackson et al., 1967	Maynard and Barker, 1972	Siciliano et al., 1975	Jackson et al., 1967	Siciliano et al., 1975
Asparagus		50			3		16
Bean: dry (navy)	68						
green	440		250	150	100	200	270
lima	310		130			88	27
Beet	2,600	1,300	1,700	2,600	3,000	550	510
Broccoli		2,300		940			
Brussels							
sprouts							
Cabbage	200	1,200	320	720	780		84
Carrot	66	320	18	140	72	200	97
Cauliflower	230	2,000	53				250
Celery	1,500	2,200	2,800	2,400	2,200		
Corn	37						45
Cucumber	160				24		
Eggplant	1,500				300		
Endive					60		
Kale/collard			1,900		1,600	1,500	2,800
Leek	440						
Lettuce ^b	1,700	1,100	660	750	1,200		
Melon	38	500					
Mushroom							
Okra					63		70
Onion	230		180	60	2		80
Parsley	1,100		1,700				
Peas	25		40			62	20
Pepper: sweet			200		62	130	50
Potato: white	77	63	57	190	120 ^c	130	150
sweet	66		53	0			
Pumpkin or squash	690		300		460		410
Radish	1,800	740	1,500	1,800	2,700		
Rhubarb		3,200				390	
Spinach ^b	1,900	1,600	240	2,300	2,200	670	2,100
Tomato	120	0	72	89			
Turnip	1,000				2,200	1,600	3,500

^aExpressed as mg/liter (ppm) in juice, not as mg/kg fresh weight.

^bLettuce and spinach varieties whose leaves have wrinkled edges often contain higher nitrate levels than smooth-edged varieties (Barker et al., 1974; Maynard and Barker, 1972, 1974; Olday et al., 1976), but exceptions occur (Maynard et al., 1976).

^cData from Heisler et al., 1973.

TABLE 5-5B

Nitrate Content of Vegetables from Local Retail Markets

NOTE: Some of the concentrations have been rounded off to two significant figures

Vegetable	Concentration of Nitrate, mg/kg Fresh Weight, Except for Column Entitled "Class" ^a					Ranges		
	Averages		Fresh, Canned and/or Frozen		Class ^d Corr ^e and Breimer, 1979	Fresh, Canned, and/or Frozen (literature surveys)		Corr ^e and Breimer 1979
	(literature surveys)		(literature surveys)					
	White, 1975	FDA, 1979	Corr ^e and Breimer, 1979 ^b	Lee, 1970 ^c	White, 1975	White, 1975	White, 1975	
Asparagus	21	200	66	1	16-50	13-700		
Bean: dry (navy)	13 ^d	69			13 ^d			
green	250	330	430	3	200-270	44-1,100		
lima	54	220			88-130			
Beet	2,800	2,700	2,100	5	600-4,500	100-8,100		
Broccoli	780	1,900	700	2	400-860	140-1,300		
Brussels sprouts			118					
Cabbage	640	550	400 ^e	3	150-1,700	0-170		
Carrot	120	130	280	3	50-500	0-2,700		
Cauliflower	550	600	420	2	100-1,200	0-2,800		
Celery	2,300	1,800	2,300	5	50-3,200	0-4,500		
Corn	45				45	50-5,300		
Cucumber	24	160	190	2	24	17-570		
Eggplant	300	300	240	2	300	180-300		
Endive		1,400	1,300	4		10-3,800		
Kale/collard		1,900	790	3	650-4,800	30-5,500		
Leek			510	4		36-4,500		
Lettuce ^f	850	1,100	2,600	5	300-6,000	90-13,000		
Melon	430	430	290	2	280-1,200	40-600		
Mushroom		110	160	1	430	40-600		
Okra						40-400		
Onion	130	160	200	2	62-180	0-2,300		
Parsley		1,300	1,000	4		0-4,100		
Peas	28		27	1	20-62	0-110		
Pepper: sweet	120	170	120	1	50-200	0-350		
Potato: white	120	110	110	1	57-190	0-1,000		
sweet	53	64	39	1	53	0-130		
Pumpkin or squash		300	380	3	400-2,300	34-2,200		
Radish	410	1,700	1,900	5	850-6,500	60-9,000		
Rhubarb		3,200	2,100	4		400-5,500		
Spinach ^f	1,900	1,800	1,700	5	2,300-4,500	2-6,700		
Tomato	62	64	53	1	240-2,400	0-170		
Turnip		1,100	390	2	0-89	10-2,900		
Turnip greens			6,600	5	50-3,500	6,600		

^aClassification of vegetables according to nitrate content of the fresh product: 1 = most values lower than 200 mg/kg; 2 = most values lower than 500 mg/kg; 3 = most values lower than 1,000 mg/kg; 4 = most values lower than 200 mg/kg; 5 = most values lower than 500 mg/kg.

or acidic, organically rich (peat) soils lead to elevated nitrate con

The above factors increase nitrate levels in produce by affecting one or more processes. Most importantly, they affect nitrogen uptake, nitrogen transport, and nitrate reduction and assimilation. Although nitrate uptake by the roots is not strongly affected by photosynthesis, the reduction and assimilation of nitrate are closely coupled with photosynthesis, which occurs predominantly in the leaves of the plant. Photosynthesis is also required for the transfer of nitrate from its predominant location in storage vacuoles to an active metabolic pool capable of maintaining nitrate reductase levels (Aslam et al., 1976; Ferrari et al., 1973; Heimer and Filner, 1971; Martinoia et al., 1981; Shaner and Boyer, 1976). Nitrate reductase is a molybdenum-containing, intricately regulated enzyme complex that affects nitrate assimilation (Beevers and Hageman, 1969; Haynes and Goh, 1978; Wright and Davison, 1964). Thus, perturbations in nitrate reductase activity, in photosynthesis, or in nitrogen uptake can lead to nitrate accumulation.

Factors affecting nitrate accumulation are not mutually exclusive and often operate in concert to increase nitrate levels. An extreme example of interactions, involving tomatoes grown under artificial circumstances, has been reported by Luh et al. (1973). Tomatoes from plants grown at daytime temperatures of 35°C stored nitrate at approximately 0.4 mg/kg fresh weight whether nitrogen fertilizer was added or not. Tomatoes from plants grown at daytime temperatures of 20°C stored nitrate at approximately 0.7 mg/kg when denied heavy fertilization, but stored about 73 mg/kg when supplied with excess nitrogen nutrition. Although such high levels may not be found in the field, this example illustrates the importance of multiple factors in producing wide variations among field-grown crops (Corré and Breimer, 1979; Maynard et al., 1976). Thus, knowledge of how nitrate content is influenced by the above-mentioned factors can be used to lower the content of this ion in vegetables (Table 5-6). Other reviews also contain suggestions on how this may be accomplished (Hartman, 1981; National Academy of Sciences, 1978).

Li et al. (1980) and Schuphan (1974) have observed an inverse correlation between nitrate and ascorbate concentrations in vegetables. These observations support the earlier findings of Kilgore et al. (1964) who reported that turnip greens from unshaded plants exposed to normal levels of nitrogen-containing fertilizer contained nitrate at 1,593 mg/kg and ascorbate at 1,351 mg/kg, whereas shaded plants fertilized with an excess amount of nitrogen-containing fertilizer and sodium nitrate, contained nitrate at 4,707 mg/kg and ascorbate at 833 mg/kg (Kilgore et al., 1964), resulting in nitrate: ascorbate ratios of 0.30 and 0.06, respectively.

TABLE 5-6

Procedures for Reducing Nitrate Concentrations in Spinach^a

NOTE: These concentrations have been converted from nitrate-nitrogen to nitrate ion and some have been rounded to two significant figures.

Specific Adjutment	Nitrate Concentration, mg/kg			Reference
	Original Condition	Following Adjustment	Reduction in Nitrate Concentration, %	
Use of smooth-leaved (cv. Tuftegard) instead of savoyed-leaved (cv. Bloomsdale)	7,400	2,000	73	Cantliffe, 1972
50% NH ₄ -N and 50% NO ₃ -N, instead of 100% NO ₃ -N	90,000	60,000	34	Mills et al., 1976
Nitrapyrin used with 1:1 NH ₄ -N:NO ₃ -N fertilizer	60,000	40,000	34	Mills et al., 1976
Harvest after 12 hours of light instead of 0 hours	5,100	3,700	27	Minotti and Stankey, 1973
Petiole removal cv. America	2,400	1,700	29	Olday et al., 1976

Fresh-Weight Basis:

that their data "do not reflect any tendency for substantial change in the nitrate-nitrogen concentration of vegetables over the period studied." Although there are other possible explanations for the observed differences (e.g., differences in assay methods), Table 5-7, based on data from Corr  and Breimer, indicates that there may have been increases in the nitrate content of some vegetables, including carrots, lettuce, and spinach, during the past decade. If, in fact, such increases in the nitrate content have occurred, heavy use of nitrogen fertilizers, as well as the timing of application of the fertilizer, may have been a contributing factor.

TABLE 5-7

Comparison of the Nitrate Content of Fresh Market Vegetables
in the 1960's and the 1970's

NOTE: Some of these concentrations have been rounded off to two significant figures.

Vegetable	Average Concentration (mg/kg) and Number (N) of Values That were Averaged			
	Corr� and Breimer (1979)			
	1960's		1970's	
	N	Concentration	N	Concentration
Bean: green	4	400	5	450
Beet	5	1,500	12	2,300
Brussels sprout	2	25	4	190
Cabbage	7	385	23	420
Celery	1	2,800	11	2,300
Carrot	5	130	19	330
Cucumber	3	240	8	180
Endive	3	1,100	18	1,300
Leek	3	450	6	560
Lettuce	5	1,100	33	2,800
Pea	2	<5	3	41
Pepper: sweet	3	140	4	110
Potato: white	5	88	12	120
Radish	4	1,300	14	2,100
Spinach	11	1,100	34	1,900

400 sets of data compiled by Corre and Breimer (1979), which are also presented in Table 5-5, permitting classification of vegetables into five categories, according to nitrate accumulation.

To develop an estimate of nitrate in vegetables (Table 5-8), the committee averaged the estimates of White (1975) and Corre and Breimer (1979) given in Table 5-5. Thus, the committee's estimates reflect the large data base used by Corre and Breimer while giving some bias to U.S. products, which were surveyed by White. When one of the data bases failed to provide a figure, the committee used the average value from the other survey.

The estimates of White and those of Corre and Breimer rely primarily on nitrate levels measured in fresh vegetables (although White did include some processed vegetables in his averages). In general, the nitrate content of processed vegetables (canned or frozen) and of fresh, cooked vegetables is lower because nitrate stored in vacuoles is released during boiling into the surrounding fluid, resulting in reductions as high as 50% (Kenny and Walshe, 1975; Kilgore et al., 1963; Krehl and Winters, 1950; Sohler et al., 1976). The extent of these reductions depend on the amount of water used. Similar losses of vitamin C can also occur (Krehl and Winters, 1950). In industrial processing, the most important procedure affecting nitrate content is blanching, which usually decreases the nitrate (Corre and Breimer, 1979). Blanching of spinach can lead to a 30% decrease in nitrate content; however, more than 90% of the ascorbate content can be lost during this process (Schuphan, 1974). Additional industrial processing may decrease the nitrate content further. For example, there is a 40% to 50% reduction of nitrate in spinach following canning (Lee et al., 1971; Sohler et al., 1976). Freezing also appears to decrease the level of nitrate (Corre and Breimer, 1979). For nitrate-rich vegetables, Corre and Breimer (1979) estimated that a nitrate loss of 20% to 25% on a fresh weight basis was a reasonable average.

Quantitative determination of nitrite in vegetables is difficult because some of the nitrite is lost during extraction (Klepper, 1979). As a result, the reported data may underestimate the true nitrite content by a factor (approximately 2) that varies with each vegetable (Klepper, 1979). Although reports of the nitrite content of vegetables are sparse (Corre and Breimer, 1979), it is known that the concentration of nitrite in fresh market vegetables is generally low and usually does not exceed 1 to 2 mg/kg (Corre and Breimer, 1979). Older data reflect higher levels of nitrite -- from 4.3 to 76 mg/kg (Richardson, 1907), although the reasons for this are unknown.

The nitrite content of vegetables is known to increase with

Vegetable	Concentration, mg/kg (fresh weight)	
	Nitrate ^a	Nitrite ^b
Artichoke	12 ^c	0.4
Asparagus	44	0.6
Bean: green	340	0.6
lima	54	1.1
dry (navy)	13	NR ^d
Beet	2,400	4.0 ^e
Broccoli	740	1.0
Brussels sprouts	120	1.0
Cabbage	520	0.5
Carrot	200	0.8 ^e
Cauliflower	480	1.1
Celery	2,300	0.5
Corn	45	2.0
Cucumber	110	0.5
Eggplant	270	0.5
Endive	1,300	0.5
Kale/collard	800	1.0
Leek	510	NR ^d
Lettuce	1,700	0.4
Melon	360	NR ^d
Mushroom	160	0.5 ^e
Okra	38 ^c	0.7
Onion	170	0.7
Parsley	1,010	NR ^d
Peas	28	0.6
Pepper: sweet	120	0.4
Potato: white	110	0.6
sweet	46	0.7
Pumpkin and squash	400	0.5
Radish	1,900	0.2
Rhubarb	2,100	NR ^d
Spinach	1,800	2.5 ^e
Tomato	58	NR ^d
Turnip	390	NR ^d
Turnip greens	6,600	2.3

^aData are primarily from fresh vegetables and are derived by averaging the data of White (1975, 1976) and Corr   and Breimer (1979).

^bData are primarily from processed vegetables, adapted from Siciliano et al. (1975).

^cData from Siciliano et al., 1975.

^dNR = No data reported

of processed vegetables, probably since nitrate is released from vacuoles where it is normally sequestered (Martinoia et al., 1981). The soluble nitrate is then available for reduction to nitrite. Thus, the nitrite content of processed and even frozen vegetables is often from two- to threefold higher than that of their unprocessed counterparts (Corré and Breimer, 1979; Siciliano et al., 1975). Elevated levels are also characteristic of processed infant foods (Kamm et al., 1965).

Average amounts of nitrite in fresh and/or processed vegetables are given in Table 5-8. On the basis of these data, Siciliano et al. (1975) concluded that the nitrite content of commercial fresh, frozen, or canned vegetables, as available to the consumer, is generally low (1.0 mg/kg or less). However, these investigators also pointed out that prolonged storage of open, thawed, cooked, or uncooked vegetables or their storage under improper conditions, may lead to higher nitrite levels through the conversion of nitrate to nitrite.

The nitrite content is drastically increased, exceeding 100 mg/kg and reaching approximately 400 mg/kg, in vegetables pickled by fermentation, which is traditional in some areas of Japan and China (Matsui, 1944; Yanagihara et al., 1963). Such foods do not constitute a significant source of nitrate or nitrite intake per capita in the United States, although they could be a substantial component of the diets of some ethnic groups within this country. The nitrite content of U.S.-style pickles does not seem to have been measured.

Another important factor affecting nitrite concentration is the level of ascorbate in vegetables since ascorbate can react with and eliminate nitrite (Lemoigne et al., 1937; Mirvish et al., 1972) (also see discussion in Chapters 4 and 6). Table 5-9 presents the ratio of ascorbate to nitrate, on a molar basis, for those vegetables listed in Table 5-9. The data in Table 5-9 indicate that the ratio of ascorbate to nitrate for one class of vegetables may vary by at least fivefold, depending upon plant growth conditions, and may vary more than 30-fold from one vegetable to another (e.g., celery versus artichoke). The low ascorbate concentration in carrots may explain why nitrite accumulates so readily in temperature-abused carrot juice (Hall et al., 1977; Keating et al., 1973), even though the nitrate content of carrots is generally not high (Table 5-9).

Plants also contain antioxidants other than ascorbate. For example, polyphenols occur in especially large amounts in many plants and plant products. Although most phenols inhibit nitrosation, some may enhance it (see Chapter 4).

Fruits and Fruit Juices: Nitrate and Nitrite. White (1975)

NOTE: Some of the concentrations have been rounded off to two significant figures

Vegetable	Nitrate, mg/kg ^b	Ascorbate, mg/kg ^c	Ascorbate, mg/kg ^d	Ascorbate, mg/kg ^e	Ratio of Ascorbate ^e to Nitrate ^b mol/mol
Artichoke	12		90	120	3.5
Asparagus	44		840	330	2.7
Bean: green	340	90	760	190	0.20
lima	54		290	290	1.9
dry (navy)	13		60	0	--
Beet	2,400		100	100	0.02
Broccoli	750		1,100	1,100	0.50
Cauliflower sprouts	120	1,020	4,200	1,000	3.1
Cabbage	520			470 ^f	0.32
Carrot	200	26	220	80	0.14
Chamomile flower	480	520	780	780	0.57
Celery	2,300		90	100	0.02
Corn	45		120	120	0.94
Cucumber	110		340	120	0.39
Eggplant	270		50	50	0.06
Endive	1,300		100	100	0.03
Kale/collard	800		1,200	1,200	0.56
Leek	510		180	170	0.12
Lettuce	1,700		80	130	0.03
Melon	360			320	0.31
Mushroom	160		50	30	0.07
Okra	38			310	3.0
Onion	170	90	100	100	0.21
Parsley	1,000	2,700	1,700	1,700	0.60
Peas	28		220	270	3.4
Pepper: sweet	120	1,200	1,300	1,300	3.7
Potato: white	110	110	730	200 ^h	0.62
sweet	46		210	210	1.6
Pumpkin and squash	400	105	140	150	0.13
Radish	1,900		260	260	0.05
Rhubarb	2,100		90	90	0.02
Spinach	1,800	840	510	510	0.10
Tomatoes	60		200	230	1.4
Turnip	390		360	360	0.33
Turnip greens	6,600		1,400	1,400	0.07

Ascorbate levels are for fresh, uncooked produce as purchased. Ascorbate loss during cooking varies with the method, but generally ranges from 15% to 60% (Diem and Lentner, 1970; Pennington and Church, 1980). Data from Table 5-8.

Data from Kelly and Latzko, 1980.

Data from Diem and Lentner, 1970.

Data from Pennington and Church, 1980.

Ascorbate concentrations average 510 mg/kg for freshly harvested, 420 mg/kg for stored, cabbage.

Average of cantaloupe, honeydew, and watermelon.

Values range from about 260 mg/kg in recently harvested potatoes to about 120 mg/kg after 3 months storage and about 80 mg/kg after 6 months storage (Pennington and Church, 1980).

Huguet et al. (1976) found pears to contain nitrate at 34 mg/kg (or mg/liter of juice), whereas cherries and apples contained 24 mg/kg. These levels were essentially unaffected by fertilizer usage. Different varieties may have different nitrate levels, but the significance of these differences is difficult to assess from the limited data presented (Huguet et al., 1976). Kenny and Walshe (1975) detected no nitrate in Golden Delicious apples. Nitrite has been measured less frequently. In one report, Harada et al. (1972) detected less than 1 mg/kg of nitrite in apples, oranges, and other fruits.

Because of the higher levels of nitrate in fruit reported in studies that were not included in White's survey, the committee has used an estimate of twice the amount used by White--20 mg/kg nitrate--and assumed as did White that the nitrite content of fruits is negligible (Table 5-3).

Baked Goods and Cereals: Nitrate and Nitrite. The accumulation of nitrate in grains is increased by many of the same factors that influence the accumulation of nitrate in vegetables (see section on vegetables above and the detailed discussion in Corré and Breimer, 1979); however, accumulation is generally less in grains than in stems and in leaves (Hanway et al., 1963; Wu and McDonald, 1976). The nitrate and nitrite content of cereals can also increase during drying if internal-combustion-type dryers are used (Fornal et al., 1975). White (1975) drew attention to the absence of reliable data concerning nitrate and nitrite contents of U.S. baked goods, a situation that prevails today. He did report that bread contained nitrate at approximately 20 mg/kg and nitrite at approximately 0.17 mg/kg, based on two limited surveys (Richardson, 1907; Rooma, 1971).

Studies by McNamara et al. (1971) indicate that the nitrate content of winter wheat seeds varies with strain and growth conditions (0.4 to 11 mg/kg), indicating that a reasonable median for nitrate in winter wheat might be approximately 2 mg/kg. Similar data were obtained in studies of a variety of wheat cultivars (Wu and McDonald, 1976). These investigators reported nitrate concentrations of approximately 9 to 15 mg/kg for wheat and 4 to 14 mg/kg for wheat flour. Selenka and Brand-Grimm (1976) found that wheat flour contained nitrate at approximately 1 mg/kg and nitrite at 1.2 mg/kg, but white bread and flour after baking contained nitrate at an average of 13 mg/kg and nitrite at 3.4 mg/kg. Darker breads formulated with rye (e.g., rye bread and pumpernickel) averaged nitrate concentrations of 20 mg/kg and nitrite at 4.3 mg/kg. Harada et al. (1972) reported that flours contain approximately 3 mg/kg nitrite, whereas bread crumbs, noodles, and macaroni contain concentrations between 10 and

To estimate the nitrate and nitrite content of baked goods and cereals, the committee averaged the data presented above for nitrate (excluding graham crackers) to obtain an average of 12 mg/kg. For nitrite, the value for white and dark breads and White's earlier estimate were averaged to obtain a concentration of 2.6 mg/kg (Table 5-3).

Milk and Milk Products: Nitrate and Nitrite. When White (1975) estimated the nitrate intake from milk, he used data from Hänni (1954), Davis and MacDonald (1953), and Sander (1967), which indicate that milk contained nitrate in concentrations less than 1 mg/liter. In 1964, Wright and Davison reported that milk from control cows in a nitrate feeding study contained 4.8 mg/liter. Recent data from Denmark further suggest that levels of nitrate in milk may be higher than previously believed, and the State Food Institute (Statens Levnedsmiddelinstitut, 1981) has estimated that the average nitrate concentration of milk and milk products is 8 mg/liter.

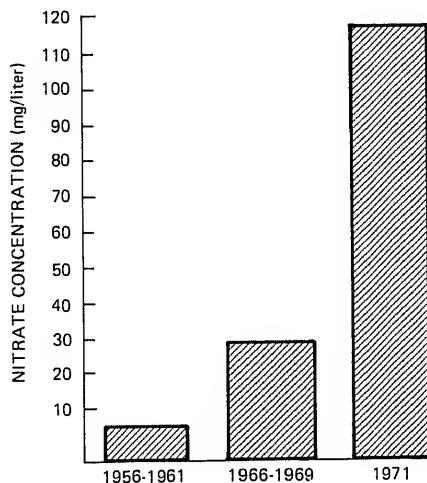
The concentrations of both nitrate and nitrite ions may reflect the nitrate content of feed and forage available to the ruminant or the nitrate intake of the nursing mother. There is a trend toward the use of increasing amounts of nitrogen fertilizer, which is capable of increasing the nitrate load of forage crops (ap Griffith, 1958, 1960; Whitehead et al., 1978). Very high nitrate/nitrite levels can be reached under certain conditions, sometimes leading to nitrite intoxication in livestock (Case, 1957; Hanway et al., 1963; Lorenz, 1978; Whitehead et al., 1978; Wright and Davison, 1964). Although the nitrate content of milk generally does not increase drastically when cows eat fodder or drink water containing high levels of nitrate (Cefalu, 1954; Hänni, 1954; Wright and Davison, 1964), a fourfold increase of nitrate, from 4.8 mg/liter to approximately 20 mg/liter, was observed in one case when nitrate intake was high (Wright and Davison, 1964). In addition, nitrate levels in dried milk increase when direct-firing processes are used (Manning et al., 1968; Rammell and Joerin, 1972).

In some cheeses, nitrate concentrations averaged from 7 to 28 mg/kg (Pedersen et al., 1980; Woerner and Fricker, 1960) and nitrite concentrations averaged from approximately 1.2 to 1.6 mg/kg (Harada et al., 1972; Woerner and Fricker, 1960) after potassium nitrate had been added during processing to prevent bacterial growth. However, a recent report on the nitrate and nitrite content of Danish cheeses indicates that comparable levels (10 mg/kg nitrate; 0.2 mg/kg nitrite) are found in cheese regardless of whether nitrate has been added (Statens Levnedsmiddelinstitut, 1981).

Nitrate and Nitrite. The nitrate content of water is an important factor in determining the exposure of humans to nitrate because of the large quantity consumed daily and the apparently increasing concentrations of nitrate in U.S. drinking water. Concentrations of nitrate and nitrite in surface water and groundwater are dependent upon geochemical conditions and agricultural run-off as well as upon management practices in the treatment of waste from humans and animals (World Health Organization, 1978), including municipal and industrial wastewaters, refuse dumps, animal feedlots, and septic tanks (Emerick, 1974; National Academy of Sciences, 1977a)

Gradual increases in nitrate levels in many surface and groundwater sources of drinking water have been reported. Concentrations of nitrate in major U.S. rivers, recorded by the U.S. Geological Survey, indicate trends toward increasing nitrate concentrations in the Delaware, San Joaquin, Ohio, Mississippi, and Wabash Rivers (National Academy of Sciences, 1977a). Figure 5-1 shows nitrate concentrations in the Sangamon River, a source of drinking water in central Illinois. At times during 4 months of 1971, Sangamon River water contained nitrate concentrations close to 200 mg/liter. This high level probably resulted from fertilizer run-off (Commoner, 1977; Klepper, 1978; Kohl et al., 1971).

The amount of fertilizer used is not the sole factor affecting nitrate run-off from agricultural practices. In some areas, irrigation practices play an important role as well (Saffigna and Keeney, 1977; Spalding et al., 1980). Furthermore, an estimated 1.6 billion metric tons of animal excreta annually contribute nitrate to soils and waters in the United States (Garman, 1969).



(Durfor and Becker, 1964), in which the average concentration was 0.66 mg/liter. However, many sources of drinking water have been reported to contain higher concentrations of nitrate. The Safe Drinking Water Committee (National Academy of Sciences, 1977a) cited several examples of water supplies containing nitrate in concentrations exceeding 44 mg/liter. The source of most of these supplies was well water, although municipal supplies were also implicated. Table 5-10 presents data from additional surveys of water with high nitrate content.

In estimating the intake from water, the committee has used the 1962 survey of Durfor and Becker (1964), as did White (1975). However it has increased the average to 1.3 mg/liter because of the data just presented, which demonstrate increases in nitrate concentration since 1962 and higher concentrations in smaller water supplies. The committee recognizes that this average concentration underestimates the exposure of persons living in a region with high-nitrate drinking water. Therefore, it has also estimated the intake from water with a nitrate concentration of 100 mg/liter (comparable to the average amount measured in the Sangamon River). Nitrite content is considered to be negligible (Table 5-3); however, this may not be true in regions of other countries where water with high levels of nitrate is stored, resulting in reduction of nitrate to nitrite and subsequent ingestion of high nitrite levels (Li et al., 1980).

Tobacco

Nitrate. Tobacco is rich in nitrate, the amount depending on the strain. The highest concentrations of nitrate are found in the Burley and Maryland varieties. The amount and type of fertilizer, location of leaves on the plant, and curing process (air-cured versus flue-cured) are also important determinants of nitrate content of tobacco (Fuqua et al., 1974; Mizusaki et al., 1977a,b; Sims et al., 1970, 1979; Wynder and Hoffmann, 1968). Many other factors that influence the accumulation of nitrate in vegetables (discussed earlier) probably also influence the nitrate content of tobacco leaves.

The contribution of tobacco to the exposure of humans to nitrate and/or nitrite is difficult to determine because tobacco is used in many different ways and amounts. For example, individuals who chew tobacco probably extract, ingest, and retain large amounts of nitrate. Exposure from this use does not seem to have been investigated and no estimates have been developed by the committee. On the other hand, although cigarette smokers do not directly ingest tobacco nitrate as nitrate, reliable data on intake indicate that nitrogen oxides formed during tobacco combustion are inhaled. See section

Year(s)	Nitrate Concentration, mg/liter	Systems Containing These Concentrations/ Total	Reference
1942-1974	> 44	79/1467 ^a	Woll, 1978
1945-1951	4-40	11 ^b	Harmeson <u>et al.</u> , 19
1950	> 44	183/389 ^c	Bosch <u>et al.</u> , 1950
1950	>440	51/389 ^c	Bosch <u>et al.</u> , 1950
1950	> 22	28/514 ^d	Bosch <u>et al.</u> , 1950
1950	> 44	16/514 ^d	Bosch <u>et al.</u> , 1950
1960-1961	> 21	182/800 ^e	Anonymous, 1969
1960-1961	> 45	88/800 ^e	Anonymous, 1969
1963	> 45	4/789 ^f	Larson, 1963;
1965	> 45	37 ^g	Ridder and Oehme, 1974
1965	> 90	3 ^g	Ridder and Oehme, 1974
1966	> 44	481/8844 ^b	Harmeson <u>et al.</u> , 19
1966-1970	11-90	30 ^h	Larson and Henley, 1966
1969	> 45	19/969 ⁱ	McCabe <u>et al.</u> , 1970

^aSurvey of groundwater samples in Maryland.

^bMaximum nitrate runoff from selected watersheds in Illinois.

^cSurvey of wells in Minnesota, including 139 wells supplying families for which cases of methemoglobinemia had been reported.

^dSurvey of municipal water supplies in Minnesota.

^eSurvey of wells in southern California.

^fSurvey of public groundwater supplies in Illinois.

^gSurvey of municipal water supplies in Kansas.

^hSurvey of wells in Illinois.

ⁱSurvey of public water supplies throughout the United States.

Air

Atmospheric Concentrations of Nitrogen Oxides. The most complete and authentic source of data on nitrogen oxide concentrations is the National Aerometric Data Bank of the U.S. Environmental Protection Agency (EPA). This data bank receives inputs from the National Air Sampling Network (NASN) as well as from other state and local sources. The NASN is comprised of approximately 100 sites at which nitrogen dioxide and sulfur dioxide are monitored. Until recently, it also included six Continuous Air Monitoring Project (CAMP) stations. Additional data have been provided by more localized studies, such as the Chattanooga Study (Helms et al., 1970) and the California Air Resources Board (1974), which issued a 10-year summary of data gathered through its large network of monitoring stations.

The distribution of nitrogen oxides in the atmosphere is by no means uniform. Localized concentrations often exceed the "average" concentration by a factor as high as 100 (U.S. Environmental Protec-

where nitrogen acid plants and uncontrolled stationary combustion sources produce oxides of nitrogen. The effect of these sources on pollutant concentrations is largely determined by the movement of the air mass containing the pollutants.

Geographic and meteorologic factors can combine to amplify the effect of man-made emissions. For example, in the calm air mass of the Los Angeles basin, both horizontal and vertical movements of the air mass are minimal. Under these conditions, nitrogen oxides build up, and nitric oxide is converted to the more harmful nitrogen dioxide.

Because a large portion of urban nitrogen oxides is generated by human activity, variations in oxide concentrations correlate directly with such activities. The major variable is vehicular traffic. Periods of heavy traffic, such as morning and evening rush hours, produce correspondingly high concentrations of nitric oxide. During slack periods of traffic, such agents as breezes and sunlight disperse, convert, or otherwise reduce these high concentrations. These competing factors create typical daily patterns in nitrogen oxide concentrations.

Tables 5-11 and 5-12 show typical nitric oxide and NO_x (nitric oxide plus nitrogen dioxide) levels in California from 1963 to 1972. Virtually all of the data were collected with the original Greiss-Saltzman procedure (Saltzman, 1954) (see methods section of this chapter). Table 5-13 provides the nitrogen oxide concentrations in 47 U.S. cities. These data were accumulated with the chemiluminescence and sodium arsenite methods. Although the results of the two methods are similar, they cannot be compared closely because sampling locations and analysis periods were different. Nonetheless, the data are probably typical of the nitrogen dioxide concentrations in the various cities. Thus, nitrogen dioxide levels are generally below the national air quality standard: an annual arithmetic mean of 0.05 ppm ($\sim 90 \mu\text{g}/\text{m}^3$) (U.S. Environmental Protection Agency, 1971b). However, at peak periods, urban air can contain nitrogen oxides in concentrations as high as 1 ppm. To estimate average exposure, the committee averaged the concentrations for nitrogen dioxide given in Table 5-13 for chemiluminescence assays to give a value of $58 \mu\text{g}/\text{m}^3$. For high nitrogen oxide intakes, the committee has used $118 \mu\text{g}/\text{m}^3$, the value for Los Angeles.

Concentrations of Nitrogen Oxides in Indoor Air. Indoor combustion sources give rise to high concentrations of a number of pollutants, especially carbon monoxide and nitrogen dioxide. Space heaters and water heaters appear to emit pollutants into building interiors only when their design is faulty or when the appliance has not been main-

Hourly Average Concentration of Nitric Oxide in California^aAverage Concentration, $\mu\text{g}/\text{m}^3$, 25°C

Location	1963		1964		1965		1966		1967		1968		1969		1970		1971		1972	
	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max
Anaheim	26	360	31	370	31	860	26	860	490	50	810	31	1,600	74	1,190	59	1,090	80	1,000	91
Azusa	25	520	23	260	20	290	21	320	25	490	37	360	26	430	42	500	43	500	42	790
Burbank	124	1,400	105	890	123	1,260	115	1,110	127	1,110	131	1,620	156	1,780	167	1,350	160	1,370	150	1,130
Fresno	13	1,070	23	580	18	640	16	660	21	730	21	780	23	1,050	—	—	—	—	—	—
La Habra	—	—	—	—	—	—	—	—	—	—	62	1,130	41	570	42	620	58	583	68	980
Lennox	—	—	—	127	127	1,250	148	2,160	181	2,220	166	1,660	157	2,050	176	2,270	181	2,340	172	1,870
Long Beach	134	1,140	140	1,540	117	920	123	970	140	1,280	143	1,170	130	1,160	148	1,960	118	1,070	128	1,520
Los Angeles																				
Downtown	121	1,050	98	1,600	101	1,410	112	1,020	119	1,410	120	1,360	119	1,490	130	1,600	139	1,410	122	1,130
USC	69	700	71	1,000	70	740	73	850	74	750	—	—	—	—	—	—	—	—	—	—
West LA	101	1,050	89	1,170	92	1,290	83	1,300	88	1,330	92	1,050	89	1,840	108	1,360	111	1,720	91	1,120
Oakland	58	1,140	90	1,140	58	810	60	840	60	1,120	55	910	—	—	—	—	—	—	—	—
Oakland-Jackson	—	—	—	—	—	—	—	—	—	—	—	—	43	850	40	710	44	1,050	45	660
Pasadena	—	—	—	—	—	—	—	—	—	—	81	820	71	820	84	980	69	690	89	860
Villa Street	70	850	63	760	50	520	60	610	66	650	57	550	—	—	—	—	—	—	—	—
Pomona	—	—	—	—	68	580	75	550	95	760	105	910	103	900	118	810	121	780	116	820
Redlands	—	—	—	—	—	—	—	—	—	—	22	460	20	210	22	1,170	32	1,760	43	650
Redwood City	—	—	—	—	—	—	—	—	42	530	54	590	47	630	42	580	41	780	48	590
Richmond	—	—	—	—	—	—	—	—	46	710	60	500	52	870	32	820	31	730	65	490
Riverside	—	—	57	1,350	46	700	46	530	53	640	—	—	—	—	—	—	—	—	41	680
Sacramento	48	1,330	49	1,330	41	1,190	44	920	42	1,110	33	1,140	33	1,140	35	750	33	780	—	—
San Bernardino	11	310	11	310	27	420	39	620	41	440	42	360	26	260	39	280	52	490	41	630
San Diego	44	910	30	1,350	38	1,110	62	1,480	43	980	57	1,350	38	1,110	36	860	81	2,150	66	1,300
San Francisco	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ellis Street	—	—	—	950	—	—	—	—	—	—	70	1,600	38	740	58	630	48	690	68	620
Union Square	102	1,590	103	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mission Street	—	—	—	—	52	760	—	—	49	1,090	63	1,670	—	—	—	—	—	—	—	—
Santa Cruz County	—	—	—	—	—	—	9	160	6	160	7	200	7	250	—	—	—	—	—	—
Stockton	—	—	39	620	32	590	41	1,070	16	440	25	620	26	640	22	700	25	700	30	570

Hourly Average Concentrations of NO_x in California^a

Average Concentration, ppm

Location	1963		1964		1965		1966		1967		1968		1969		1970		1971		1972	
	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max	Mean	Max
Anaheim	0.06	0.42	0.06	0.35	0.06	0.80	0.06	0.53	0.08	0.76	0.12	0.45	0.11	1.10	0.10	1.05	0.12	1.18	0.12	1.02
Azusa	0.06	0.48	0.07	0.28	0.06	0.35	0.06	0.48	0.07	0.52	0.08	0.45	0.07	0.53	0.10	0.56	0.10	0.54	0.10	0.77
Burbank	0.16	1.20	0.14	0.83	0.17	1.18	0.16	1.02	0.19	1.08	0.25	1.72	0.22	1.73	0.23	1.30	0.22	1.22	0.20	1.16
Fresno	0.03	0.93	0.04	0.52	0.04	0.55	0.04	0.60	0.04	0.66	0.05	0.70	0.05	0.94	—	—	—	—	—	—
La Habra	—	—	—	—	—	—	—	—	—	—	0.10	0.96	0.07	0.52	0.06	0.60	0.10	0.65	0.11	1.05
Lennox	—	—	—	—	0.17	1.21	0.18	1.95	0.23	2.10	0.21	1.66	0.19	1.86	0.21	2.15	0.22	2.21	0.21	1.71
Long Beach	0.17	1.14	0.18	1.36	0.15	0.85	0.16	0.85	0.19	1.24	0.20	1.20	0.18	1.15	0.20	1.94	0.16	1.05	0.17	1.50
Los Angeles	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Los Angeles Downtown	0.16	1.11	0.14	1.46	0.16	1.35	0.17	0.96	0.17	1.34	0.17	1.27	0.16	1.41	0.18	1.50	0.20	1.42	0.18	1.16
USC	0.12	0.88	0.12	0.89	0.13	0.82	0.12	0.86	0.13	0.84	—	—	—	—	—	—	—	—	—	—
Oakland	0.10	0.95	0.11	1.26	0.08	0.74	0.08	0.79	0.09	1.06	0.08	0.85	—	—	—	—	—	—	—	—
Oakland-Jackson Pasadena	—	—	—	—	—	—	—	—	—	—	—	—	0.07	0.70	0.07	0.66	0.08	0.96	0.07	0.67
Villa St.	0.11	0.79	0.11	0.70	0.10	0.60	0.11	0.78	0.12	0.71	0.15	1.03	0.14	1.19	0.16	1.20	0.13	0.86	0.16	0.94
Pomona	—	—	—	—	0.12	0.59	0.12	0.59	0.15	0.67	0.16	0.84	0.16	0.89	0.18	0.81	0.18	0.80	0.17	0.94
Redlands	—	—	—	—	—	—	—	—	—	—	0.04	0.43	0.03	0.22	0.05	1.00	0.07	1.51	0.07	0.60
Redwood City,	—	—	—	—	—	—	—	—	0.07	0.49	0.07	0.52	0.07	0.70	0.07	0.58	0.06	0.74	0.07	0.55
Richmond	—	—	—	—	—	—	—	—	0.07	0.90	0.09	0.55	0.08	0.80	0.06	0.72	0.05	0.63	0.08	0.44
Riverside	—	—	—	—	—	—	0.08	0.80	0.07	0.67	—	—	—	—	—	0.04	0.20	0.06	0.06	0.60
Sacramento	0.08	1.19	0.81	1.22	0.07	1.05	0.07	1.00	0.07	1.14	0.05	1.06	0.06	1.03	0.06	0.72	0.05	0.74	—	—
San Bernardino	0.02	0.25	0.01	0.25	0.05	0.39	0.07	0.57	0.08	0.48	0.07	0.36	0.06	0.31	0.08	0.30	0.08	0.48	0.08	0.58
San Diego	0.06	0.96	0.04	1.18	0.05	0.98	0.08	1.42	0.05	0.84	0.07	1.66	0.05	0.94	0.05	0.73	0.10	1.82	0.09	1.14
San Francisco	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ellis Street	—	—	—	—	—	—	—	—	—	—	0.10	1.42	0.07	0.64	0.09	0.62	0.07	0.70	0.09	0.58
Union Square	0.13	1.34	0.14	0.93	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mission Street	—	—	—	—	0.08	0.71	—	—	0.07	0.97	0.09	1.50	—	—	—	—	—	—	—	—
Sanja Cruz County	—	—	—	—	—	—	0.03	0.17	0.02	0.20	0.02	0.22	0.02	0.23	—	—	—	—	—	—
Stockton	—	—	0.06	0.68	0.05	0.53	0.05	0.90	0.04	0.42	0.05	0.60	0.06	0.62	0.04	0.62	0.04	0.68	0.05	0.55

Table from National Academy of Sciences, 1977b. Based on data from State of California Air Resources Board, 1966.

Region	Average Concentration of Nitrogen Dioxide for Period of Operation, $\mu\text{g}/\text{m}^3$, 25°C	
	Sodium Arsenite Method ^b	Chemiluminescence Method
Atlanta	80	62
Baltimore	96	64
Boston	74	--
Chattanooga	53	38
Chicago ^c	117	121
Cincinnati	73	61
Cleveland	57	53
Columbus	68	52
Corpus Christi-Victoria	43	43
Dallas-Fort Worth	76	47
Dayton	64	53
Denver ^c	42	110
Detroit-Port Huron	80	60
Dubuque	30	23
Florida, Southeast (Miami)	55	53
Florida, West Central (Tampa)	56	52
Four Corners	30	--
Genesee-Finger Lakes (Rochester)	48	26
Hampton Roads (Norfolk)	52	39
Hartford-New Haven-Springfield	82	73
Houston-Galveston	64	66
Indianapolis	61	56
Los Angeles	182	118
Louisville	87	68
Massachusetts, Central (Worcester)	71	--
Memphis	64	31
Michigan, Central (Grand Rapids)	59	44
Minneapolis-St. Paul	31	47
National Capital ^c	88	64
New York-New Jersey-Connecticut	100	65
Niagara Frontier (Buffalo)	32	49
Omaha-Council Bluffs	60	30
Pennsylvania, Central (Johnstown)	25	64
Pennsylvania, South Central (Lancaster)	60	36
Pennsylvania, Southwest (Pittsburgh)	78	64
Pennsylvania-Upper Delaware Valley,		

TABLE 5-13 (Continued)

Region	Average Concentration of Nitrogen Dioxide for Period of Operation, $\mu\text{g}/\text{m}^3$, 25°C	
	Sodium Arsenite Method ^b	Chemiluminescence Method
Phoenix-Tucson	80	69
Providence	45	--
Puget Sound (Seattle)	47	51
San Diego	63	76
San Francisco Bay Area	85	84
St. Louis	79	58
State Capital (Richmond)	58	37
Toledo	54	38
Wasatch Front (Salt Lake City)	62	114
Wisconsin, Southeast (Milwaukee)	76	--

^aTable adapted from National Academy of Sciences, 1977b, pp. 86-87. Based on data from U.S. Environmental Protection Agency, 1973.

^bConcentrations corrected to reflect 85% collection efficiency. Data indicate that there is 95% confidence that the corrected measurements are within ± 10% of actual nitrogen dioxide concentrations.

^cAll measurements at same site. In other regions, all measurements were not made at same site.

In a 1973-1974 study of indoor sources of air pollutants, Cote et al. (1974) determined the emission rates of nitric oxide and nitrogen dioxide from an unvented gas-fired space heater. Under low-flame steady-state conditions, typical pollutant emissions were 214 mg/hour for nitric oxide and 130 mg/hour for nitrogen dioxide. When the flame was high, the emissions were 837 mg/hour for nitric oxide and 272 mg/hour for nitrogen dioxide.

A brief EPA study showed that peak nitrogen dioxide concentrations as high as 1 ppm ($\sim 1,880 \mu\text{g}/\text{m}^3$) and 1-hour averages ranging from 0.25 to 0.50 ppm ($\sim 470\text{-}940 \mu\text{g}/\text{m}^3$) are reached in a closed kitchen with no external ventilation (Eaton et al., 1972).

concluded that:

- Emissions from gas stoves contribute nitrogen dioxide, nitric oxide, and carbon monoxide to the indoor atmosphere of houses in which such stoves are used. Concentrations of these gases in kitchens responded rapidly to stove use and, for a given house during a given season, there was a rough correlation between average nitrogen dioxide concentrations and average stove use.

- Nitrogen dioxide and nitric oxide were produced in roughly equal amounts. Indoor concentrations of these pollutants were invariably higher than those outside.

- Normal stove operations frequently resulted in nitrogen dioxide concentrations in the kitchens averaging $100 \mu\text{g}/\text{m}^3$ over the 2-week sampling periods.

- Comparison of samples taken during warm and cold weather during 1973-1974 indicated that pollutant concentrations were more uniformly distributed within the various rooms of the house during cold weather, when the house was closed up more often than during the warmer months.

- A diffusion experiment conducted in one of the houses showed that the half-life of nitrogen dioxide was only one-third of that for carbon monoxide and nitric oxide, indicating that nitrogen dioxide decays through reaction or adsorption in addition to normal dilution from air exchange. The effect was observed in some of the other houses by comparing the relative concentrations of nitrogen dioxide and other pollutants in various parts of the house.

Moschandreas et al. (1978) conducted an extensive study of indoor air quality in houses in five U.S. metropolitan centers. These investigators identified three types of indoor environments based on whether the houses had electric cooking and heating appliances, gas furnaces and electric cooking appliances, or gas cooking and heating equipment. In houses equipped with gas cooking appliances, indoor concentrations of nitric oxide were consistently higher than those observed outdoors. Houses with gas furnaces but electric cooking appliances usually contained concentrations of nitric oxide that were higher than those found outdoors. However, during intervals interspersed throughout the monitoring period, concentrations of nitric oxide measured outdoors surpassed those measured indoors. Indoor nitric oxide concentrations in totally electric homes were usually lower than outdoor concentrations.

The influence of gas stoves on indoor levels of nitrogen

same type of monitor in an epidemiological study to show that indoor levels of nitrogen dioxide were significantly higher in homes with gas stoves than in those with electric stoves. Other possible indoor combustion sources of NO_x are gas water heaters, gas dryers, and charcoal broilers, especially if they are faulty.

There is only limited information concerning the effects of combustion sources on indoor air quality in commercial buildings. Nevertheless, the sources mentioned above can presumably affect the quality of indoor air in commercial buildings as they do in residential buildings.

In summary, the highest average concentrations of indoor nitrogen dioxide are found in domestic kitchens. These concentrations can range from 100 to ~940 $\mu\text{g}/\text{m}^3$ and may reach peaks of approximately 1,880 $\mu\text{g}/\text{m}^3$ when a gas stove is used in a poorly ventilated kitchen.

Concentrations of Nitrogen Oxides in Workplace Air. The presence of nitrogen dioxide in workplace air is fairly common, resulting primarily from the decomposition of nitrate (e.g., during dynamite blasting or silaging), from reactions of nitric acid with metals or other reducing agents (e.g., during acid dipping and dye and aniline manufacturing), from various processes in which air is heated to a high temperature (e.g., in furnaces or during welding and cutting torch operations), or from the exhaust of internal combustion engines. Although nitrogen dioxide (and/or dinitrogen tetroxide) has been used in industry as a nitrating or oxidizing agent and as the oxidizer in hypergolic rocket fuel, it is produced most often as an undesirable by-product from industrial practices and/or processes (Lewis, 1980). The National Institute for Occupational Safety and Health (1976) has estimated that at least 1.5 million workers in the United States are potentially exposed to nitrogen dioxide.

Estimates of concentrations of nitrogen oxides in various occupational settings are difficult to derive because there are generally no data. However, Wade et al. (1950) reported that 90 deaths prior to 1930 and 47 deaths between 1930 and 1949 were caused by exposure to high concentrations of nitrogen dioxide.

Concentrations of Nitrogen Oxides in Cigarette Smoke. Although originally thought to be a mixture of nitric oxide and nitrogen dioxide, the nitrogen oxides in fresh cigarette smoke, as inhaled, are predominantly, if not almost exclusively, nitric oxide (Adams et al. 1978; Jenkins and Gill 1980; Norman and Keith 1965). Nitric

oxide and its derivatives react with other molecules in cigarette smoke to form a variety of mutagens and carcinogens, such as tobacco-specific nitrosamines (Hoffmann et al., in press).

The nitric oxide content of cigarette smoke is positively related to the nitrate content of the tobacco (Broadus et al., 1965; Fuqua et al., 1976; Sims et al., 1979). In view of the factors influencing the nitrate content of tobacco, it is not surprising to find wide variations in the nitric oxide content of mainstream smoke from different cigarettes (Table 5-14).

Several methods have been used to determine the nitric oxide content of cigarette smoke (Adams et al., 1978). Jenkins and Gill (1980) reported a procedure that yields nitric oxide values that are higher than those determined by other methods: for smoke with a high nitric oxide content they were approximately 50% higher, for smoke with a low nitric oxide content they were about 170% higher. Their method involves rapid dilution to minimize interference in the assay by other smoke components. This rapid dilution might also minimize side reactions that may occur in cigarette smoke during normal inhalation. Based on the values given by these investigators for nine experimental cigarettes, the average concentration of nitric oxide per cigarette is approximately 510 μg . The committee has used 0.51 mg nitric oxide per cigarette to estimate the possible exposure of humans from this source (Table 5-3).

Summary

Wide ranges of nitrate and nitrite concentrations can be found in all food categories surveyed by the committee. For this reason, the committee has often based its estimates of the concentrations of these substances on data from broad surveys and, when possible, has averaged data from a number of studies. Canadian surveys of cured meat products have shown that the concentration of nitrate has been decreasing for the past decade, presumably because of the elimination of nitrate salts in all but a few products in Canada. Although there are no published data for the nitrate content of cured meat products in the United States, a similar decline is assumed to have occurred in U.S. cured meat products as well because a voluntary program to reduce the use of nitrate has been adopted by the meat industry. Current average nitrate concentrations in U.S. cured meat products have been estimated by the committee to be approximately 40 mg/kg (expressed as the nitrate ion). The committee estimated that the average concentration of residual nitrite in cured meats was 10 mg/kg (expressed as the nitrite ion). It arrived at this value by decreasing the average reported by Kolari and Aunan (1972) by 70% to reflect residual nitrite

Estimates of the Nitrogen Oxide Content of Cigarette Smoke

NOTE: Some numbers presented in this table have been rounded off to two significant figures.

Nitrogen Oxides, μg /Cigarette	Average	References
Range		
--	150	Bokhoven and Niessen, 1961
--	270 ^a	Gori, 1976
260-420	340	Adams <u>et al.</u> , 1978
--	280 ^b	Hoffmann <u>et al.</u> , 1980
--	100 ^c	Hoffmann <u>et al.</u> , 1980
90-1,400	510 ^d	Jenkins and Gill, 1980

^aNo data are available for average nitrogen oxide content of cigarettes before 1960; however, an experimental cigarette considered to be representative of cigarettes marketed at that time contained 270 μg .

^bAverage amount in smoke of regular retail brands of cigarettes available in 1980.

^cAverage amount in smoke of low-tar retail brands of cigarettes available in 1978-1979.

^dAverage amount in smoke of nine types of experimental cigarettes. Higher recovery method gave higher values for nitrogen oxides.

cured meat products. Based on a limited number of studies of nitrate and nitrite content in fresh meat, 10 mg/kg nitrate and 1 mg/kg nitrite were assumed to be the average concentrations in these products.

Vegetables also vary in nitrate and nitrite content. Nitrate content can be modified by growing conditions, by time of harvest, by certain genetic factors, and by the amount and kind of nitrogen fertilizer used and the timing of its application. An overall average concentration of nitrate and nitrite in vegetables was not estimated. Instead, the committee developed averages for 35 different vegetable types. The nitrate values, which are primarily for fresh vegetables, are based on literature surveys conducted by White (1975) and Corré and Breimer (1979). Most of the concentrations of nitrite in vegetables used by the committee were derived from the study by Siciliano et al. (1975), which was based primarily on concentrations in processed vegetables.

Average concentrations developed by the committee for nitrate in other food sources are: fruit, 20 mg/kg; baked goods and cereals, 12 mg/kg; and milk products, 0.5 mg/liter. The average nitrite content was estimated to be 2.6 mg/kg in baked goods and cereals and negligible in the other two categories.

The concentration of nitrite is also considered to be negligible in water; however, an estimated average concentration of nitrate in U.S. drinking water is 1.3 mg/liter, based primarily on a 1962 survey of the 100 largest U.S. municipal drinking water supplies and adjusted to reflect reports of increases in water supplies since 1966 and the higher nitrate content of smaller drinking water supplies.

Varying concentrations of nitrogen oxides are found in air outdoors, indoors, and in the workplace. In general, atmospheric levels of nitrogen dioxide are less than 0.05 ppm ($\sim 90 \mu\text{g}/\text{m}^3$), although as much as 1 ppm of nitrogen oxides during peak periods. In domestic kitchens, peak levels of nitrogen dioxide may also reach concentrations of 1 ppm ($\sim 1,880 \mu\text{g}/\text{m}^3$). Estimates of average concentrations in occupational settings are difficult to derive because of the lack of data.

Cigarette smoke also contains nitrogen oxides -- primarily nitric oxide. The committee has concluded that 0.51 mg of nitric oxide per cigarette, an average based on the measurements of Jenkins and Gill (1980), is a reasonable estimate of exposure from this source.

A summary of the estimated average concentrations (with the exception of vegetables) is given in Table 5-3. These values are used in the following section to estimate the relative importance of each environmental source to total exposure of humans to nitrate and nitrite and are not intended to represent absolute amounts contained in the individual sources.

EXPOSURE OF HUMANS TO NITRATE, NITRITE, AND NITROGEN OXIDES

The following discussion of the ingestion of nitrate and nitrite takes into consideration the relative contributions of a variety of exogenous sources such as air, water, and food, including cured and fresh meats. However, daily intake of nitrate and nitrite (especially when expressed as averages for an entire population) may not be as relevant to the determination of in vivo effects as peak concentrations that may occur at certain body sites immediately after ingestion. In addition, estimates of nitrite ingestion do not include

of the mean exogenous exposure of the U.S. population to nitrate and nitrite as well as estimates for certain population groups whose exposure may deviate significantly from the mean.

Exposures from Food

Two methods are generally used to estimate the ingestion of nitrate and nitrite in food. One method depends on direct assays of nitrate and nitrite in duplicate portions of representative meals. The other method involves the use of published tables of average consumption, e.g., the Household Consumption Survey of the U. S. Department of Agriculture (1980), and the nitrate and nitrite content of various dietary constituents reported in the literature.

The direct assay method affords several advantages over the method using food consumption tables: The nitrate and nitrite content in food is measured after it has been prepared, thereby providing a more accurate assessment of the amounts actually ingested. Food consumption is also measured directly, thus eliminating the need for food consumption tables that can over- or underestimate actual intake (Selenka and Brand-Grimm, 1976).

One weakness in the assessment of intake by the direct assay method is the possibility of biasing the data through selection of meals to be tested, since individual variations in dietary habits occur on a daily and seasonal basis. As shown in Table 5-15, more

TABLE 5-15

Per Capita Ingestion of Nitrate and Nitrite
Measured By Direct Assay of Meals

NOTE: Some of the values have been rounded off to two significant figures.

Amount Ingested, mg/Person/Day				Country	References
Nitrate		Nitrite			
Average	Range	Average	Range		
50	26-81	3.7	0.6-7.3	Sweden	Jägerstad and Nilsson, 1976 Jägerstad et al., 1976

study conducted in Sweden (Jägerstad and Nilsson, 1976; Jägerstad et al., 1976). In contrast, Stephany and Schuller (1978) recorded a twentyfold range in nitrite ingestion and a fortyfold range in nitrate ingestion in the Netherlands. The wide ranges in the latter study were attributed to seasonal variations in the diets. Thus, intake figures from a direct assay could be misleading.

In this report, data on exposure to nitrate and nitrite from food were obtained exclusively from food consumption/production tables and averages developed from published reports of nitrate and nitrite content, primarily because direct assay data for meals consumed by the U.S. population are not available. The committee has used consumption data from the U.S. surveys listed in Table 5-16 to estimate the average per capita consumption of various categories of foods (Table 5-17). In an attempt to compensate for the wide ranges in consumption, it has also estimated the intake for populations groups with high (4 times the average) consumption of cured meat and high (4 times the average) consumption of vegetables.

Despite the limitations of the data on average consumption as well as those described for environmental concentrations in the preceding section, the committee believes that its estimates are a useful indication of relative exposures of the human population to nitrate and nitrite from the various environmental sources.

Cured Meats. Four estimates of average individual daily intake of nitrate and nitrite from cured meats are presented in Tables 5-18 and 5-19. Some of these data differ by approximately elevenfold. Such discrepancies are due to different estimates of residual nitrate and nitrite in products as they are consumed, coupled with different estimates of average consumption.

In Table 5-18, White's estimate of 9.4 mg daily nitrate intake from cured meats reflects the nitrate content of European meat product in 1972 (White, 1975, 1976). This estimate is also based on production figures for 1972, rather than on consumption data, and it may overestimate intake. More recently, Birdsall (1981) also used White's value as a conservative (maximal) estimate of nitrate content. The Food and Drug Administration's (1979) estimate of zero nitrate in cured meats does not take into account the fact that even uncured meats contain nitrate.

Hartman (1981) used White's estimate of 9.4 mg/person/day to calculate the intake of nitrate from cured meats (Table 5-18). However, he reduced White's estimate by 20% to account for a drop in the nitrate content of cured meats since 1972 and to account for a

Consumption, g/Person/Day, by Study

	United States			United Kingdom		Norway		Federal Republic of Germany	
	White, 1975, 1976 ^a	U.S. Department of Agriculture, 1980 ^b	Food and Drug Administration, 1979 ^c 1980 ^b	Ashton, 1970	Walker, 1975	Høyem, 1974		Selenka and Brand-Grimm, 1976	
meats, meats, including veal and lamb	45	20	29 30	--	--	--	--	--	--
processed meats, including luncheon meats	180 ^d	80 ^d	120 ^d 120 ^d	31	32	120	110		10
beef	150	--	55 --	64	66	100	89		10
potatoes	120	--	27 --	--	140	230	130		5
tablets and capsules	270	200	82 320	--	200	330	220		15
oats	180	170	-- 290	--	--	--	150		15
cereals and milk	90	62	-- 83	--	--	190	160		11
products	500	310	-- 300	--	--	550	12		30

ates based on farm production figures correction for estimated losses in food preparation.

ates based on food consumption surveys.

ates' based the Fourth Natural Household Menu Census Study conducted from July 1972 to June 1973 by the National Health and Medical Research Corporation of America, Northbrook, Illinois.

on the assumptions that cured meats constitute roughly one-half of all processed meats and that processed meats constitute roughly one-half of total meat consumption (see text discussion on meats). Therefore, estimates of meat consumption have been multiplied by 4 to estimate total meat consumption.

An Estimate of Average Daily Intake

<u>Source</u>	<u>Amount</u>
Cured meats	30 g
Fresh meats	60 g
Vegetables	190 g
Fruits	220 g
Baked goods and cereals	100 g
Milk and milk products	370 g
Water	1.6 liters
Air	20 m ³
Cigarettes	20 cigarettes

(40 mg/kg) of nitrate in cured meat products also takes into account the decrease in residual nitrate. It averaged the consumption data of White (1975, 1976), the U.S. Department of Agriculture (1980), and the Food and Drug Administration (1980) (Table 5-16) to develop an estimate of approximately 30 g/person/day for cured meats. Thus, average consumption would be 1.2 mg of nitrate per person per day (Table 5-20).

Nitrite ingested in cured meats was estimated to be 0.30 mg/person/day (30 g/person/day x 10 mg/kg nitrite) (Table 5-21). This estimate is close to those generated independently by Birdsall (1981) and Hartman (1981) (Table 5-19).

Although the residual nitrite content of many cured meats is low, cured meats can contribute a substantial percentage of the nitrite ingested by certain subgroups of the population that consume large amounts of these products. Thus, the committee has estimated the intake for a subgroup with a high cured meat diet in addition to estimates for average intake from meats. Assuming that daily consumption of cured meats in this subgroup is 4 times the average consumption (120 g/person/day), daily intake of nitrate would be 4.8 mg and nitrite intake would be 1.2 mg.

Fresh Meat Products. Average consumption of fresh meats has been estimated by averaging the consumption of "total meats" indicated by U.S. surveys in Table 5-16 and dividing by 0.5 (approximately one-half of meat consumed is fresh, unprocessed meat). This figure is approximately 60 g/person/day. Assuming a 10 mg/kg concentration of nitrate in fresh meats, daily intake of nitrate

Four Estimates of Per Capita Daily Dietary
Nitrate Intake by U.S. Residents

NOTE: Some of these data have been rounded off to two significant figures.

<u>Intake, mg/Person/Day, and (Percentage of Total Daily Intake from All Sources), by Study</u>				
<u>Food</u>	<u>White, 1975, 1976</u>	<u>Food and Drug Administration, 1979</u>	<u>Hartman, 1981</u>	<u>Birdsall 1981</u>
Cured meats	9.4 (9.4)	0 ^a (0)	0.43 (0.6)	9.4 (9.4)
Vegetables	86 (86)	37 (95)	62.0 (91.7)	86 (86)
Fruits and juices	1.4 (1.4)	NR ^b	1.2 (1.8)	1.4 (1.4)
Bread	2.0 (2.0)	NR	2.0 (2.8)	2.0 (2.0)
Milk and milk products	0.2 (0.2)	NR	0.2 (0.3)	0.2 (0.2)
Water	0.7 (0.7)	2 (5)	2.0 (2.8)	0.7 (0.7)
TOTAL	100 (100)	39 (100)	70 (100)	100 (100)

^a0 indicates a dietary contribution that is relatively unimportant in comparison with other food categories listed.

^bNR = No data were reported.

Four Estimates of Per Capita Daily Dietary
Nitrite Intake by U.S. Residents

NOTE: Some of these data have been rounded off
to two significant figures.

<u>Intake, mg/Person/Day, and (Percentage of Total Daily Intake from All Sources), by Study</u>				
<u>Food</u>	<u>White, 1975, 1976</u>	<u>Food and Drug Administration, 1979</u>	<u>Hartman, 1981</u>	<u>Birdsall, 1981</u>
Red meats	2.4 (92)	0.22 (65)	0.37 (68)	0.35 (61.5)
Vegetables	0.2 (7)	0.12 (35)	0.16 (29)	0.2 (35)
Fruits and juices	0 ^a (0)	NR ^b	0 (0)	0 (0)
Lead	0.02 (1)	NR	<0.02 (3)	0.02 (3.5)
Milk and milk products	0 (0)	NR	0 (0)	0 (0)
Other	0 (0)	0 (0)	0 (0)	0 (0)
TOTAL	2.6 (100)	0.34 (100)	0.54 (100)	0.57 (100)

^a indicates a dietary contribution that is relatively unimportant
in comparison with other food categories listed.

NR = No data were reported.

Committee's Estimates of Per Capita Daily Nitrate Intake for the U.S. Population (Averaged) and for Three Population Subgroups, Indicating the Spectrum of Ranges of Intake Believed to be Prevalent in the United States Today

Source	Exposure, mg/Person, and Percentage Contribution from Each Dietary Source ^a							
	U.S. Average		High Cured Meat ^b		Vegetarian ^c		Nitrate-Rich Water ^d	
	mg	%	mg	%	mg	%	mg	%
Cured meats	1.2	1.6	4.8	6	0	0	1.2	0.5
Fresh meats	0.6	0.8	0.6	0.8	0	0	0.6	0.2
Vegetables	65	87	65	83	260	97	65	28
Fruits and juices	4.3	6	4.3	6	4.3	1.6	4.3	1.8
Baked goods and cereals	1.2	1.6	1.2	1.5	1.2	0.4	1.2	0.5
Milk and milk products	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.1
Water	2	2.6	2	2.5	2	0.7	160	68
TOTAL	75		78		268		233	

^aThese data pertain to exposure to exogenous sources (ingestion) only. Analysis of impact of body load of nitrite and nitrate is to be found in Chapter 8. Figures are crude and are given for the purpose of determining relative contributions from various sources (see text).

^bAssuming 4 times the daily consumption of cured meats listed for the "average" daily ingestion.

^cAssuming a vegetarian consumes 4 times the amount of vegetables in the same distribution as does the "average" person.

^dData are for the Sangamon River area in Central Illinois.

Committee Estimates of Per Capita Daily Nitrite Intake for the U.S. Population (Averaged) and for Three Population Subgroups, Indicating the Spectrum of Ranges of Intake Believed to be Prevalent in the United States Today

Source	Exposure, mg/Person, and Percentage Contribution from Each Dietary Source ^a							
	U.S. Average		High Cured Meat ^b		Vegetarians ^c		Nitrate-Rich Water ^d	
	mg	%	mg	%	mg	%	mg	%
Cured meats	0.30	39	1.2	71	0	0	0.30	39
Fresh meats	0.06	7.7	0.06	3.5	0	0	0.06	7.7
Vegetables	0.12	16	0.12	7	0.48	62	0.12	16
Fruits and juices	0.01	1.3	0.01	0.6	0.01	1.3	0.01	1.3
Baked goods and cereals	0.26	34	0.26	15	0.26	34	0.26	34
Milk and milk products	0.01	1.3	0.01	0.6	0.01	1.2	0.01	1.3
Water	0.01	1.3	0.01	0.6	0.01	1.3	0.01	1.3
TOTAL	0.77		1.7		0.77		0.77	

^aThese data pertain to exposure to exogenous sources (ingestion) only. Analysis of impact of body load of nitrite and nitrate is to be found in Chapter 8. Figures are crude and are given for the purpose of determining relative contributions from various sources (see text).

^bAssuming 4 times the daily consumption of cured meats listed for the "average" daily ingestion.

^cAssuming a vegetarian consumes 4 times the amount of vegetables in the same distribution as does the "average" person.

^dData are for the Sangamon River area in Central Illinois.

of Agriculture on "average portion sizes." White (1975) estimated the average total vegetable consumption to be approximately 270 g per person daily, whereas the FDA estimated only 80 g per person per day. If white potatoes are excluded from the lists, then the FDA estimate is 55 g and White's estimate of 150 g is nearly 3 times greater. The estimates diverge even more for nitrate-rich vegetables (categories 4 and 5, Table 5-5). The FDA estimated the individual daily intake of these vegetables to be 6.8 g, whereas White estimated 33 g per person per day. This is more than a fivefold difference.

Because there are inherent biases in each method used (e.g., production figures even when corrected for loss often result in overestimates and food consumption surveys may over- or underestimate actual consumption), the committee has averaged these two sets of data (Food and Drug Administration, 1979; White, 1975) to estimate the average consumption listed in Table 5-22. Based on these averages, the contribution of nitrate from vegetables in the average U.S. diet is estimated to be approximately 65 mg nitrate and 0.12 mg nitrite per person per day (Table 5-21). These figures can be compared with previous estimates given in Tables 5-18 and 5-19. If vegetarians consume, on the average, 4 times the amount of the same vegetables as the general population, then vegetarians will ingest 260 mg of nitrite and 0.48 mg of nitrite per day (Tables 5-20 and 5-21).

Fruits and Fruit Juices. The committee estimates that the daily per capita ingestion of fruits and fruit juices is 216 g. This is the average of three estimates (Food and Drug Administration, 1980; U.S. Department of Agriculture, 1980; White, 1975) (Table 5-16). Assuming that these products contain nitrate concentrations of approximately 20 mg/kg, which is double White's 1975 value for fruits, the average ingestion is estimated to be 4.3 mg of nitrate per person per day. Nitrite intake from this source is very low -- .01 mg per person per day (Tables 5-20 and 5-21).

Baked Goods and Cereals. Ingestion of bread, rolls, and other baked goods is estimated to be 78 g per person per day -- the average value of three separate U.S. consumption estimates (Food and Drug Administration, 1980; U.S. Department of Agriculture, 1980; White, 1975) (Table 5-16). Cereal consumption is estimated to be 22 g per person per day (Food and Drug Administration, 1980). Thus, total average consumption of baked goods and cereals is estimated to equal 100 g/person/day. Based on this estimate of consumption and the estimated nitrate and nitrite content of the products in this food category, the committee estimates that 1.2 mg of nitrate and 0.26 mg of nitrite are ingested per person per day from this source (Tables 5-20 and 5-21).

Vegetable	Concentration, mg/kg		Average Amount of Vegetable Consumed Per Day, g	Average Consumption Per Day, μ g	
	Nitrate	Nitrite		Nitrate	Nitrite
Artichoke	12	0.4	0.12	1	0
Asparagus	44	0.6	1.2	53	0
Bean: green	340	0.6	8.1	2,700	4
lima	54	1.1	1.4	76	1
dry (navy)	13	NR ^a	3.8 ^b	49	1
Beet	2,400	4.0	1.3	3,100	5
Broccoli	740	1.0	1.5	1,100	1
Brussels sprouts	120	1.0	0.2	24	0
Cabbage	520	0.5	5.5 ^c	2,800	2
Carrot	200	0.8	5.8	1,200	4
Cauliflower	480	1.1	0.9	440	1
Celery	2,300	0.5	3.8	8,800	1
Corn	45	2.0	14	610	27
Cucumber	110	0.5	1.9	210	1
Eggplant	270	0.5	0.4	110	0
Endive	1,300	0.5	0.01	18	0
Kale/collard	800	1.0	0.5	400	0
Leek	510	NR ^a	NR ^a	(5) ^d	1
Lettuce	1,700	0.4	13	22,000	5
Melon	360	NR ^a	11 ^b	3,900	NR
Mushroom	160	0.5	0.3	49	0
Okra	38	0.7	NR ^a	1	0
Onion	170	0.7	6.8	1,100	4
Parsley	1,010	NR ^a	NR ^a	(10) ^d	
Peas	28	0.6	6.2	170	3
Pepper: sweet	120	0.4	1.4	170	0
Potato: white	110	0.6	73	8,300	4
sweet	46	0.7	3.4	160	2
Pumpkin and squash	400	0.5	1.4	560	0
Radish	1,900	0.2	0.3	580	0
Rhubarb	2,100	NR ^a	NR ^a	(21) ^d	
Spinach	1,800	2.5	1.8	3,200	4
Tomato and products	58	NR ^a	21	1,200	
Turnip	390	NR ^a	0.2	78	
Turnip greens	6,600	2.3	0.3	2,000	0
TOTAL			~190	~65,000	~120

^aNR = No data reported.

^bNot on the FDA (1979) list. Figure is one-half of White's (1975) estimate.

^cIncluding sauerkraut.

^dNitrate intake per day based on average daily consumption of 0.01 g of vegetable.

considered to be 370 g per person per day for U.S. adults. This is an average of three separate U.S. consumption estimates (Food and Drug Administration, 1980; U.S. Department of Agriculture, 1980; White, 1975) (Table 5-16). Based on estimates that these products contain 0.5 mg/liter of nitrate and negligible levels of nitrite, intake would be approximately 0.2 mg of nitrate and less than 0.01 mg of nitrite per person per day from this source (Tables 5-20 and 5-21). However, if the concentration of nitrate in U.S. milk and milk products is similar to that found in Danish products (e.g., an average of 8 mg/liter), intake could be somewhat higher -- approximately 3.0 mg per person per day. Even if this higher concentration were present, the amount ingested would not contribute a significant percentage of daily nitrate intake for the average adult.

For infants, however, milk with a nitrate content of 8 mg/liter can contribute substantially to exposure since consumption of 0.75 liter of such milk by a 4.5-kg infant would be comparable, on a body-weight basis, to daily ingestion of approximately 120 mg of nitrate by an average adult. Turek *et al.* (1980a,b) found that nitrate present in the urine of infants was an accurate reflection of the amount of nitrate ingested. Suckling infants excreted appreciable concentrations of nitrate (averaging 39 mg of nitrate per liter) and bottle-fed infants excreted approximately 3 times as much. Although breast milk or cow's milk could contribute to nitrate intake by infants, extremely high intakes occur most frequently when infant formula is prepared with well water containing high concentrations of nitrate (> 44 mg/liter) (Walton, 1951). (See also discussion of methemoglobinemia in Chapter 9.) For example, Donahoe (1949) reported that the cyanosis of a breast-fed infant with presumed methemoglobinemia cleared when the mother stopped drinking well water suspected of containing a high concentration of nitrate.

Collectively, these observations indicate that levels of nitrate that could be significant to the infant can be accumulated in the milk of both humans and ruminants. Infant formulas made with high-nitrate water may also be an important source of nitrate (see next section).

Exposures from Water

The major ingredient of most beverages (e.g., coffee, tea, and soft drinks) is water. The average daily consumption of water from these sources plus drinking water is approximately 1.6 liters of water for the average adult (National Academy of Sciences, 1977a) (Table 5-17). The committee has estimated that U.S. drinking water supplies contain an average nitrate concentration of 1.3 mg/liter.

To estimate intake for regions with drinking water supplies containing high concentrations of nitrate, the committee used data from studies of the Sangamon River region near Decatur, Illinois, where concentrations of nitrate in water averaged approximately 100 mg/liter in 1971 (Kohl et al., 1971). Assuming that 1.6 liters are ingested daily, intake in such regions would be approximately 160 mg per person per day (Table 5-20)

Exposures from Air

Atmospheric Nitrogen Oxides. Inhaled nitrogen oxides and alkyl nitrites could play an important role in the exposure of humans to nitrate and nitrite (Ehrenberg et al., 1980; Erlandsson, 1981; Hoffmann et al., 1975; Newmark and Mergens, 1981; World Health Organization, 1978). Parks et al. (1981) have found that considerable amounts of nitrate and nitrite may be accumulated in the lungs under certain circumstances and that these ions may be rapidly and widely distributed in the body as nitrate. Very recently, Oda (1981) reported that inhalation of nitrogen dioxide leads to the appearance of relatively large amounts of nitrate and nitrite in the blood of rats. Pryor and Lightsey (1981) reported that nitrogen dioxide reacts with unsaturated fats to produce nitrite. This reaction could occur in vivo as well.

An average concentration of nitrogen dioxide in the atmosphere is usually less than the air quality standard of $90 \mu\text{g}/\text{m}^3$, although concentrations of nitrogen oxides in air of smog-laden cities may reach 1 ppm. Estimates of the daily intake of nitrite and nitrate from this source range widely. Erlandsson (1981) estimated that an atmospheric NO_x concentration of $114 \mu\text{g}/\text{m}^3$ in Gothenburg, Sweden, would result in an average daily exposure of 1.2 mg nitrate and 0.9 mg nitrite. At maximal NO_x concentrations of $929 \mu\text{g}/\text{m}^3$, he estimated the average daily exposures to be 9.4 mg nitrate and 7 mg nitrite. In the United States, Newmark and Mergens, 1981, observed that intake can be as high as 1 mmol (average of 54 mg nitrite plus nitrate) in cities during periods of smog formation.

Although the figures pertaining to inhalation exposure are conjectural and in need of confirmation, the committee, for purposes of demonstration, has assumed that all of the nitrogen dioxide is converted to nitrate (this will result in a high estimate since it is likely that less than 100% will be converted) and that the average adult breathes 20 m^3 of air per day. If, the average nitrogen dioxide concentration is $58 \mu\text{g}/\text{m}^3$, the estimated average intake of nitrate from this source is 1.5 mg. High intake can be estimated by using $118 \mu\text{g}/\text{m}^3$ (the average in Los Angeles) to give 3.1 mg nitrate/day. Peak exposures could occur during heavy smog formation and result in

the amount ingested. Also, for purposes of demonstration, the committee has assumed that 20 cigarettes (one pack) is smoked daily and that 100% of the mainstream smoke is inhaled, although the percent actually inhaled varies from individual to individual.

Under the conditions just described, if all of the nitric oxide inhaled were converted to nitrate, a concentration of 0.51 mg of nitric oxide per cigarette would result in the ingestion of approximately 21 mg of nitrate per 20 cigarettes. This is a high estimate because the conversion of nitric oxide to nitrate is probably less than 100%. Modern, low-tar U.S. cigarettes would contribute one-half to one-third the amount of nitric oxide. Small cigars may produce from 0.2 to 2.0 mg of nitric oxide per cigar (Adams et al., 1978) and could contribute significantly to exposure, even if only a small portion of the smoke is inhaled.

Summary

Tables 5-20 and 5-21 present estimates of the nitrate and nitrite intake for the average U.S. population, for those whose diets contain higher-than-average amounts of vegetables or meats, and for those who are exposed to nitrate-rich drinking water. Actually, the intake of nitrate and nitrite by the average U.S. citizen will probably vary. For example, the amount of meats and vegetables consumed generally varies on a daily and seasonal basis. Additionally, although the committee did not include an estimate of the nitrate intake from the atmosphere in these summary tables, it is likely that inhalation of nitrogen oxides will contribute to the total body burden of nitrate and nitrite and exposure to smog is at least an occasional experience for most Americans. The data in Tables 5-20 and 5-21 can be used to generate further estimates for various population subgroups based on combinations of the types of exposure just mentioned.

These estimates indicate that the average U.S. population is exposed to nitrate primarily from vegetables (87%). Other contributors of nitrate intake are, in descending order of importance: fruits and juices (6%), water (~3%), and cured meats (~2%). Intake of nitrite is provided by cured meats (39%), baked goods and cereals (34%), and vegetables (16%). It is important to point out, however, that these are estimates of the intake and not necessarily the endogenous exposure since 50% of ingested nitrate is converted to nitrite in vivo. Contributions of various sources to the total body burden of nitrite, which takes this fact into account, are given in Chapter 8.

throughout this chapter, not the least of which are limitations inherent in the analytical methods used to measure the nitrate, nitrite, and nitrogen oxides in various media. Also, because estimates of intake from foods were based on food consumption tables rather than on direct assay, the committee's estimates may reflect any inherent errors in these data. Thus, the estimate of 0.12 mg of nitrite (16%) from vegetables as a U.S. average is subject to error of uncertain dimensions because the standard errors cannot be calculated for the nitrite concentration in each vegetable presented in Table 5-8.

These estimates are probably most useful as indicators of the relative contributions made by various sources to the average daily intake. That is, although they are crude, the individual estimates in this section do suggest that some sources of nitrate and nitrite ingestion are more important than others. Additionally, they point out some ranges in exposure that are probably characteristic of large segments of the U.S. population today. For example, they indicate that approximately 39% of ingested nitrite comes from cured meats and that consumption of much larger amounts of cured meats, as in the case of a high cured meat diet, can contribute significantly (71%) to the total nitrite intake from exogenous sources.

Data in Table 5-22 indicate that only a few vegetables contribute most of the nitrate load. In addition, evidence presented earlier in Table 5-7 suggests that heavy use of nitrogen fertilizers during the past decade may have caused significant increases in the nitrate content of carrots, lettuce, and spinach.

Drinking water is an important consideration in determining environmental exposure to nitrate because of the large amount of water ingested daily and because of the reports of high concentrations of nitrate found in certain water supplies, such as private wells. Evidence that the nitrate content of drinking water may be increasing also suggests that this source of nitrate may be even more important in the future. There seems to be general agreement that the nitrite content of drinking water supplies is uniformly low and not an important contributor to human exposure to nitrite in the United States.

The committee's estimates are conjectural because there are no data on actual conversion of nitrogen dioxide to nitrate and nitrite in humans; however, nitrogen oxides may contribute to the exposure of humans to nitrate, especially in polluted atmospheres, and from tobacco smoke, which may contribute up to 21 mg of nitrate daily for persons smoking 20 cigarettes/day.

concentrations, and in methods for the accurate determination of food consumption, make it difficult to estimate precisely the exposure of humans to these compounds. Nevertheless, the committee has made rough quantitative estimates of such exposures. These estimates should not be taken at face value; rather, they should be used as a guide to gain an understanding of the relative contributions of different sources to the exposure of humans to nitrate and nitrite.

Differing lifestyles and dietary habits can lead to variations in the amount of nitrate ingested by different groups. In the average diet, vegetables contribute most of the nitrate (87% of total daily intake). Vegetarians may consume substantially higher amounts of nitrate than does the general population. Milk generally contains very low levels of nitrate; however, recent data from Denmark suggest that nitrate may be present in milk at levels higher than those previously reported. Milk is not a significant contributor of nitrate for adults, but it may be an important source of nitrate for infants. Other sources of intake include nitrate-rich drinking water and fruit and fruit juices.

Of the total daily intake of dietary nitrite, 39% is contributed by cured meats, 34% by baked goods and cereals, and 16% by vegetables. The concentration of nitrite in these foods, especially in cured meats, varies widely, and, depending on lifestyle and dietary habits, the fraction of daily nitrite exposure from any one source can vary from 0 to 90%. Thus, there may be considerable variation in the total daily intake of nitrite. (The total gastric exposure to nitrite, which includes nitrite resulting from the reduction of nitrate in the saliva, is discussed in Chapter 8.)

Two additional factors should be considered when determining the significance of exposure to nitrate and nitrite. First, vegetables contain inhibitors of nitrosation, such as ascorbic acid, and catalysts, such as certain phenols. These tend to affect the extent of in vivo nitrosation and, thus, the synthesis of N-nitroso compounds. Second, assays for the residual nitrite content of processed meats do not necessarily indicate the amount that can participate in nitrosation reactions in vivo (e.g., some species of bound nitrite may not be measured).

source is relatively small for the average U.S. population. However, peak levels of nitrogen oxides in smog-laden cities may result in more substantial exposures.

RECOMMENDATIONS

1. Methods and Data Availability. The committee recommends that more accurate estimates of exposure to nitrate and nitrite be obtained by improving assay procedures, especially to distinguish between free and bound nitrite in meat products and to examine whether residual nitrite is a true measure of nitrosating capacity. There is also a need for new analyses of nitrate and nitrite content of meats -- cured, fresh, and processed without the addition of nitrite. In addition, a better data base for the nitrate and nitrite content of milk, milk products, and grains is needed.

Furthermore, prepared meals in the United States should be analyzed for their nitrate and nitrite content. (This has already been done for representative meals in other countries.) The results should be compared with those from food consumption tables to cross-check the validity of different methods of estimating intake, as well as to provide a realistic indication of extremes in individual consumption.

To ensure effective dissemination of data regarding the nitrate and nitrite content of U.S. foods and drinking water, research staffs of the FDA, EPA, and USDA should be encouraged to expedite publication of new data in scientific journals.

2. Reduction of Nitrate Intake from Vegetables and Water. Vegetables and water containing high concentrations of nitrate are major contributors to the intake of nitrate. Thus, attention should be directed toward the feasibility of reducing the nitrate content of these sources.

Numerous approaches could be used to decrease nitrate concentrations in vegetables. These are discussed earlier in this chapter (see Table 5-6). Exposure of humans to nitrate and nitrite that can participate in nitrosation reactions can be effectively reduced by ingesting vegetables containing high levels of nitrosation inhibitors such as ascorbate. Thus, the committee recommends that further studies be conducted to develop additional methods of reducing nitrate in vegetables while retaining ascorbic acid and other inhibitors of nitrosation.

that the sources of nitrate be determined and that efforts be made to reduce these concentrations to more acceptable levels.

3. Reduction of Nitrite Intake. To the extent possible, nitrite intake should also be decreased. If the amount of nitrite added to cured meats is reduced, this reduction should not compromise protection against botulism.

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CHAPTER 6

ENVIRONMENTAL DISTRIBUTION AND EXPOSURE OF HUMANS TO NITROSATABLE SUBSTRATES AND MODIFIERS OF NITROSATION REACTIONS

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ENVIRONMENTAL DISTRIBUTION AND EXPOSURE OF HUMANS TO NITROSATABLE SUBSTRATES AND MODIFIERS OF NITROSATION REACTIONS

Exposure of humans to nitrosating agents (nitrate, nitrite, and nitrogen oxides) may contribute to the formation of N-nitroso compounds in vivo if nitrosatable substrates such as amines and amides are also present. Thus, data on the environmental distribution and exposure of humans to these nitrosatable substances will provide further indication of the potential for in vivo nitrosation reactions. Chemical agents that may inhibit or enhance the in vivo formation of N-nitroso compounds must also be considered when determining the overall potential for in vivo nitrosation. Thus, a review of data concerning the environmental distribution and exposure of humans to the various chemicals that may participate in, enhance, or block the formation of N-nitroso compounds is also included in this chapter.

NITROSATABLE SUBSTRATES: AMINO COMPOUNDS AND RELATED SUBSTANCES

Amino compounds are a diverse group of chemicals whose reactivity with nitrosating agents varies considerably among the different compounds (see Chapter 4) and also varies according to the environmental medium containing the compounds (e.g., air, food, or drugs). Most secondary amines, N-alkylureas, and N-alkylcarbamates react readily with nitrite to produce N-nitroso compounds, whereas primary, tertiary, and quaternary amines, simple N-alkylamides, and N-alkylguanidines usually react much more slowly to form N-nitroso compounds. The environmental distribution of these compounds is reviewed in this section as are data suggesting that amino compounds in the environment can react with nitrosating agents to form N-nitroso compounds.

The ubiquity and large numbers of amino compounds in the environment precludes a review of all such chemicals in this report. Thus, for discussion purposes, this chapter provides examples of the major amino compounds present in each environmental medium. For the same reasons, the committee has made no attempt to estimate the exposure of humans to these compounds. Also, since most of the reports in the literature pertain to volatile amines, the discussion will focus on exposure to this class of amines. However, certain non-volatile amines are also nitrosatable and may be important in understanding total exposure of humans to nitrosatable substances.

Food. Food is a major source of a variety of nitrosatable amines (Table 6-1). Keay and Hardy (1972) described a gas-liquid chromatographic method for the separation of dimethylamine and trimethylamine in fish as a means for monitoring spoilage since these were the major amines present. In addition to these two compounds, volatile amines including methylamine, ethylamine, n-propylamine, and isopropylamine were found in uncured pork by Patterson and Mottram (1974), who reported that the concentrations of dimethylamine, trimethylamine, n-propylamine, and isopropylamine increased during the manufacture of bacon. Patterson and Edwards (1975) reported that dimethylamine and trimethylamine reached sufficiently high concentrations in spoiled pork meat to lead to the formation of nitrosodimethylamine (NDMA).

Among the amines that have been measured in pork bellies are the free amino acids -- proline, alanine, isoleucine, leucine, methionine, phenylalanine, tyrosine, valine, glutamic acid, cysteine, and aspartic acid -- as well as hydroxyproline and N-methylglycine (sarcosine). The concentration of most of the amino acids (especially proline) increases during storage (Lakritz et al., 1976). Proline, hydroxyproline, and N-methylglycine can be nitrosated to form nitrosamines. For example, Janzowski et al. (1978) suggested that nitrosohydroxyproline is probably formed in foods from hydroxyproline during processing with nitrate and/or nitrite. However, there is no evidence that N-nitroso compounds formed from amino acids are carcinogenic (see Chapter 9).

Fujinaka et al. (1976) found detectable levels of methylguanidine in fish and meat products consumed in the Japanese diet. They estimated that the daily intake was approximately 125 μ g. The authors speculated that the precursor of methylguanidine might be creatinine or creatine, both of which are abundant in meats. High concentrations of a related guanidine derivative, agmatine, were found in the muscles of fresh abalone and top-shell and in dried squid (Kawabata et al., 1978).

Recently, Mirvish and Cairnes (1981) reported that creatinine was the precursor to methylurea formed in a Japanese dried bonito fish. In this study, the nitrosation of creatinine followed by a denitrosation reaction resulted in the formation of methylurea, possibly via nitrosomethylurea. High concentrations of creatine (3 to 6 g/kg) and its dehydration product creatinine (150 to 200 mg/kg) are found in fresh pork and beef (Vělišek et al., 1975). Nitrosation products of the reaction of creatinine with sodium nitrite under acidic conditions were identified as creatinine-5-oxime and 1-methylhydantoin-5-oxime, and nitrososarcosine was formed from creatine (Archer et al., 1971). A dried fish product and fried bacon were found to contain creatinine at 2 to 3 g/kg (Mirvish and Cairnes, 1981). Since these nitrosation reactions occurred with a very large molar excess of

nitrite treated black pepper and paprika, respectively. The NMR in pepper was probably derived by the nitrosation of the tertiary amine piperine. However, the obvious precursors, pyrrolidine or piperidine, have not been found in these spices. Singer and Lijinsky (1976a) have reported that piperidine and pyrrolidine are present in milk and meat products.

Polyamines such as cadaverine, putrescine, spermidine, and spermine were found in the germs of cereals such as barley, rice, oats, corn, wheat, and sorghum (Moruzzi and Caldara, 1964). Analysis of amines in pork and hams indicated that the concentrations of these polyamines were higher than those of monoamines, including histamine, tryptamine, tryamine, and ethanolamine (Lakritz et al., 1975).

Maga (1978) has prepared a comprehensive review of the various amines in food.

Drugs. During the past decade, considerable attention has been directed toward the nitrosation of drugs that are secondary and tertiary amines. Examples of secondary amine drugs include phenmetrazine (an anorexic), which was nitrosated to nitrosophenmetrazine in vivo in rabbits and rats (Lijinsky and Taylor, 1976), morpholine (an anesthetic), which can be converted to nitrosomorpholine (NMOR), and piperazine (an antihelmintic that yields mono- and dinitroso piperazine). Nitrosamines produced by nitrosation of some drugs are listed in Table 6-2.

Lijinsky and Greenblatt (1972) found that aminopyrine, which is a tertiary amine used as an analgesic, reacts with sodium nitrite under mildly acidic conditions both in vivo and in vitro to produce the potent carcinogen NDMA. Lijinsky et al. (1972) reported that a number of tertiary amine drugs reacted with nitrite under acidic conditions. Among these drugs are oxytetracycline (an antibiotic), aminopyrine (an analgesic), disulfiram (an antialcoholic), nikethamide (a respiratory stimulant), and tolazamide (an oral hypoglycemic). Two tertiary amines (oxytetracycline and aminopyrine) produced high yields of NDMA, whereas the dialkylamides gave rather lower yields. Tolazamide, which is a dialkylhydrazine and a substituted urea, formed nitrosohexamethyleneimine -- a potent liver carcinogen in rats. Nitrosation of tertiary amine drugs may not proceed through the amines (Lijinsky and Singer, 1975). These same authors did report, however, that such nitrosation reactions could occur at body temperature. Furthermore, long-chain aliphatic tertiary amines give higher yields than do the short-chain compounds (Lijinsky and Singer, 1975).

TABLE 6-1

Some Amino Compounds in Food

Compound	Source	Reference
<u>Alkyl amines:</u>		
Dimethylamine	Fish meal, fish products, cereals, teas Cod Pork, bacon	Lijinsky and Epstein, 1970 Keay and Hardy, 1972 Patterson and Mottram, 1974; Patterson and Edwards, 1975
	Ham, frankfurters, evaporated milk, coffee, tea, beer, wine	Singer and Lijinsky, 1976a
Diethylamine	Fish meal, fish products, cereals, teas Pork, bacon manufacture	Lijinsky and Epstein, 1970 Patterson and Mottram, 1974
Trimethylamine	Cod Pork, bacon	Keay and Hardy, 1972 Patterson and Mottram, 1974; Patterson and Edwards, 1975
Methylamine) Ethylamine) n-Propylamine) Isopropylamine)	Uncured and cured pork, bacon manufacture	Patterson and Mottram, 1974
Dipropylamine	Fish	Singer and Lijinsky, 1976a
Methylethylamine	Coffee	Singer and Lijinsky, 1976a
Methyl-n-butyl-amine	Evaporated milk	Singer and Lijinsky, 1976a
<u>Other monoamines:</u>		
Histamine) Tryptamine) Tyramine) Ethanolamine)	Pork, cured and smoked ham	Lakritz et al., 1975
<u>Amino acids:</u>		
Proline	Pork bellies Protein	Lakritz et al., 1976 Mirvish et al., 1973

Alanine)
 Glycine)
 Valine)
 Leucine)
 Isoleucine)
 Aspartic acid)
 Glutamic acid)
 Phenylalanine)
 Tyrosine)
 Methionine)
 Cystine)

Lakritz et al., 1976

Pork bellies

Cyclic amines:

Pyrrolidine
 (also formed
 from heating
 putrescine)

Spices (including paprika)
 Cooked meat and fish, wine
 Evaporated milk, coffee, wine

Sen et al., 1973
 Lijinsky and Epstein, 1970
 Singer and Lijinsky, 1976a

Piperidine
 (also formed
 from heating)
 cadaverine

Spices (including black pepper)
 Cooked meat and fish
 Ham, evaporated and whole milk, coffee

Sen et al., 1973
 Lijinsky and Epstein, 1970
 Singer and Lijinsky, 1976a

3-Hydroxy-
 pyrrolidine

Cured meats

Janzowski et al., 1978

Morpholine

Unintentional additive to foods such as
 canned hams
 Fish, ham, frankfurters, coffee, beer,
 wine

Shank and Newberne, 1972

Singer and Lijinsky, 1976a

Polyamines:

Spermine)
 Spermidine)
 Putrescine)

Pork, cured ham, soybeans, cereals

Lakritz et al., 1975;
 Moruzzi and Caldarrera, 1964

Cadaverine

Pork, cured ham, fish, cereals

Lakritz et al., 1975;
 Moruzzi and Caldarrera, 1964

Miscellaneous:

Methylguanidine

Fresh, dried, or canned fish and meat
 Fish, beef

Fujinaka et al., 1976
 Kawabata et al., 1978

Reaction of Drugs with Nitrite^a

Drug	Use	Nitros
Aminopyrine	Analgesic	NDMA
Chlorpheniramine	Antihistamine	NDMA
Chlorpromazine	Tranquilizer	NDMA
Cyclizine	Motion sickness	NDMA
Dextropropoxyphene	Tranquilizer	NDMA
Disulfiram	Antialcoholic	NDMA
Hexahydroazepinyl- nitropropiphenone	Antihashish agent	Nitros
Lucanthone	Antischistosomiasis	Nitros
Methadone	Narcotic	NDMA
Methapyrilene	Antihistamine	NDMA
Nikethamide	Stimulant	NDMA
Oxytetracycline	Antibiotic	NDMA
Quinacrine	Antimalarial	NDEA
Tolazamide	Hypoglycemic	Nitros

^a From Lijinsky, 1974.

Scheunig and Ziebarth (1976) studied the nitrosat 30 drugs. In addition to aminopyrine, they found that structure is similar to that of aminopyrine) and piper nitrosamines when incubated with sodium nitrite in the of humans.

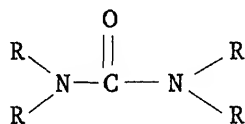
Eisenbrand et al. (1978) found concentrations of from 10 to 371 $\mu\text{g}/\text{kg}$ in 68 drugs containing aminopyrine. They related that the nitrosation of aminopyrine, which contains an amino group, might be caused by the high reactivity of with nitrogen oxides, even in the presence of ascorbic acid. Nitric oxide and nitrogen dioxide can reach levels of 1.9 mg/m^3 (1 ppm) in urban atmospheres (Chapter 5), NDMA is easily formed and accumulate on the surface of the drug.

Cyclizine has been used effectively for 5 years

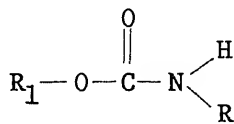
Cosmetics. Although many amines may be present in cosmetic products and are listed in the CTFA Cosmetic Ingredient Dictionary (Cosmetic, Toiletry and Fragrance Association, 1977), triethanolamine (an antiallergenic) is of special interest to the Food and Drug Administration (Wenninger, 1980) because 2-bromo-2-nitro-1,3-propanedio (bronopol), which is used as a preservative, may nitrosate this compound, resulting in the formation of nitrosodiethanolamine (NDELA), which does occur in cosmetics (see Chapter 7) (Ong and Rutherford, 1980; Schmeltz and Wenger, 1979).

In an ongoing investigation, Hecht (1981) has conducted model nitrosation studies with some typical cosmetic ingredients and sodium nitrite over a range of pH values at 37°C or 90°C. The compounds investigated included stearylalkonium chloride, lauramine oxide, dimethyl stearamine, and triisopropanolamine. Nitrosation of stearylalkonium chloride produced nitrosobenzylmethylamine (NBMA), nitrosomethylstearylamine (NMSA), and NDMA. Nitrosation of lauramine oxide produced nitrosododecylmethylamine (NDOMA), nitrosation of dimethylstearamine gave NMSA, and nitrosation of triisopropanolamine gave nitrosodiisopropanolamine (NDiLA). The yields of these reactions varied from 0.3% to 31.9%, depending on the precursor and conditions of the experiment.

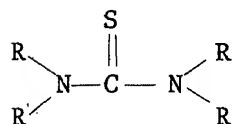
Agricultural Chemicals. Many agricultural chemicals are derivatives of alkylureas and alkylcarbamic esters, and such compounds can react with nitrite under mild acidic conditions to form N-nitroso derivatives (Elespuru and Lijinsky, 1973). Examples of these derivatives are:



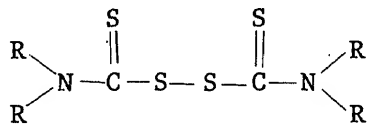
N-Alkylurea



N-Alkylcarbamate



N-Alkylthiourea



Bis(dialkylthiocarbamyl)
disulfide

such compounds as aldicarb [2-methyl-2-(methylthio)propan-1-yl [(methylamino)carbonyl]oxime], Baygon [2-(1-methylethoxy)ethyl methylcarbamate], Bux-Ten [3-(1-ethylpropyl)phenyl methylcarbamate], and 3-(1-methylbutyl)phenyl methylcarbamate], carbaryl (1-methylcarbamate), carbofuran (2,3-dihydro-2,2-dimethyl-7-methylcarbamate), Landrin (mixture of 2,3,5- and 3,4,5-trimethylcarbamate), and methomyl (N-[(methylamino)carbonyl]thioic acid methyl ester). The last compound produced footpad tumors in rats after nitrosation (Lijinsky and Schmähl, 1975).

Eisenbrand et al. (1975a) have studied the nitrosation of atrazine (2-chloro-4-ethylamino-6-isopropylamine-S-triazine), simazine (6-chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine), thiram (tetramethylthioperoxydicarbonic diamide); and Sen et al. (1975) reported the formation of NDMA from the reaction of ferbam [tris(dimethylcarbamodithioato-S,S')iron] and thioic acid 2,2-dimethylhydrazide. Nitrosation of carbaryl with nitrite occurs at pH 1, which can be reached in the stomach. Subcutaneous administration of a single high dose (1,000 mg/kg) of nitrite to Wistar rats induced local sarcomas at the site of injection (Eisenbrand et al., 1975b).

More recently, Oliver (in press) observed 0.09% to 1.0% vivo nitrosation of carbaryl by nitrite in the stomachs of pigs at pH 1.3 to 1.6, which is similar to the normal range in the human stomach. Extrapolation of these data to a 70 kg person consuming 100 g of produce containing 5 μ mol of insecticide would result in the formation of 11.2 μ g of nitrosocarbaryl. Oliver concluded that nitrite is the most probable nitrosating agent in food and that it accumulates in alkaline soils during nitrification. However, the author pointed out that the formation of nitrosamines in natural media (e.g., soil, air, and water) requires rather high concentrations of added nitrite.

In organic solvents, lipid-soluble pesticides such as organophosphates react very readily with nitrogen oxides (Mirvish et al., 1978). Therefore, nitrosation by nitrogen oxides may be more likely than nitrosation by nitrite (Mirvish et al., 1978).

Air. The amount of information regarding nitrosation in the ambient air is limited. In addition to the many urea amines that could be produced during combustion processes in lubricating oils, etc., Iqbal et al. (1980) have reported the presence of morpholine in the ambient air. Two nitrosamines -- NDMA and NMOR -- have been found in crankcase emission from diesel engines (Goff et al., 1980), and Fine et al. (1976) and F n t al. (1976) have identified NDMA as an air pollutant.

Water. Although there are no specific data concerning the presence of nitrosatable substrates in drinking water, it is likely that such amines as pesticides and herbicides are carried into water supplies by run-off from soil and, possibly, chemical dumps.

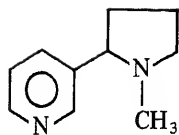
Tobacco. The occurrence of nitrosatable amines in tobacco smoke has been studied and summarized by Hoffmann et al. (1980). Precursor amines for volatile, nonvolatile, and tobacco-specific nitrosamines are derived from protein, agricultural chemicals, and alkaloids in tobacco products (Hoffmann et al., in press). Tobacco smoke also contains nitric oxide and trace amounts of nitrogen dioxide and nitrous oxide (Chapter 5). Schmeltz and Hoffmann (1977) have identified more than 600 nitrogen-containing compounds in tobacco smoke.

Singer and Lijinsky (1976b) have observed that pyrrolidine and dimethylamine were predominant among the naturally occurring secondary amines in unburned tobacco and cigarette smoke condensate. Among the volatile amines in tobacco, methylamine and aniline occur in the highest concentrations. Although Spincer and Westcott (1976) measured levels of dimethylamine and nitrogen oxides in smoke from different tobaccos, they believe that it would be difficult to make quantitative predictions of nitrosamine formation from measurements of nitrosamine precursors. Although NDEA and NPYR have been found in tobacco, their amine precursors have not been identified (Hoffmann et al., in press).

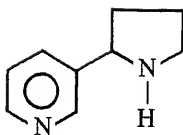
The chemical structures of some common tobacco alkaloids, including nicotine, and their concentration in cigarettes, are presented in Figure 6-1. Hoffmann et al. (in press) have shown that three of these alkaloids -- nicotine, nornicotine, and anabasine -- are nitrosated during the processing and smoking of tobacco.

Trace amounts of some aromatic amines are found in trace amounts in cigarette smoke. Among these compounds are β -naphthylamine, o-toluidine, and 4-aminobiphenyl (U.S. Public Health Service, 1979). Much higher concentrations of aromatic amines are found in sidestream smoke (smoke from the burning cigarette tip) than in mainstream smoke (smoke that passes through the cigarette) (Patrianakos and Hoffmann, 1979).

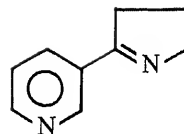
Hoffmann et al. (in press) have listed several amines, amides, and carbonates in agricultural chemicals used for the cultivation of tobacco crops. Among these compounds are dimethyldodecylamine acetate, maleic hydrazide diethanolamine, and carbaryl, which have been found in small quantities in harvested tobacco. Thus far, only diethanolamine has been studied as a possible precursor of nitrosamines in



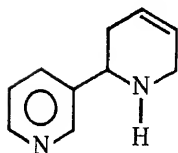
Nicotine
1,000-25,000



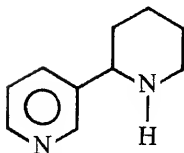
Nor nicotine
100-1,000



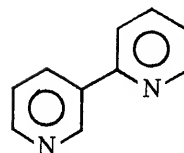
Myosmine
50-150



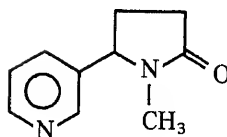
Anatabine
100-1,000



Anabasine
80-200



3,2'-Bipyridyl
10-150



Cotinine
40-200

FIGURE 6-1. Chemical structures of major tobacco alkaloids in U.S. cigarette tobacco and the concentrations ($\mu\text{g/g}$) in which they have been found in cigarettes. From Ho et al., in press.

The industrial production of nitrosamines and their precursors has been reviewed by the U.S. Environmental Protection Agency (1977). Included in this document are sources of nitrosatable amines such as methyl-, ethyl-, n-propyl-, n-butyl-, n-hexyl-, dimethyl-, and trimethylamine, which have been identified in air as pollutants resulting from the decomposition of manure from livestock, including poultry. These amines may react with nitrite in wastes or on dust or with atmospheric nitrogen oxides. Exhausts from rendering plants in which animal parts are cooked also produce such amines as ethyl-, diethyl-, and triethylamine as well as putrescine and cadaverine. Amines produced by industry include antioxidants (e.g., aryl and alkyl amines used to inhibit oxidation of lubricants), vulcanization accelerators (e.g., thiram sulfides and dialkyldithiocarbamates), pharmaceuticals, self-polishing waxes and corrosion inhibitors (e.g., morpholine), pesticides, synthetic detergents (e.g., dimethylamine), solvents (e.g., dimethylamine used in synthesis of dimethylformamide), animal glues, and photographic and leather products. The EPA report also contains production figures for amines, but the list is probably incomplete.

Analytical monitoring of occupational environments will probably lead to the identification of additional nitrosamines and their precursors. Fine (1978) has prepared a review of the exposure of humans to nitrosamines from industrial emissions and commercial products.

Endogenous Production

In addition to the amines from exogenous sources, humans are exposed to amines formed in the body. For example, bacteria in the gut can produce secondary amines from amino acid precursors (Asatoor et al., 1967; Johnson, 1977):

lysine	→	piperidine
ornithine	→	pyrrolidine
arginine	→	pyrrolidine
choline	→	dimethylamine

Other amino products may be formed by the metabolism of tryptophan in the liver and by gut flora. The amino compounds are excreted in the urine (Hill, 1980).

Endogenous nitrosatable amides include waste products such as urea, creatinine, and uric acid. These substrates might be metabolized by gut bacteria to unknown compounds. Wrong (1978) has provided evidence suggesting that creatinine is converted to a number of metabolites including methylguanidine, 1-methylhydantoin, and sarcosine.

Possible Exposure of Humans to Amino Compounds

Quantitative data on exogenous exposure and endogenous production of amines have not been adequately developed. Thus, no estimates of human exposure have been developed by the committee.

Summary and Discussion

Amino compounds that may interact with nitrite to form N-nitroso compounds are present in the environment. Foods, drugs, cosmetics, agricultural chemicals (e.g., pesticides), and tobacco products are all significant sources of nitrosatable amino compounds. Amines may also be present in drinking water, although this subject has not been specifically studied. Amines are probably not present to a significant extent in the air, except in certain environments such as animal feedlots. In addition to exogenous sources of amines, nitrosatable amino compounds are synthesized in vivo.

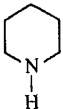

Although there are no quantitative data on exogenous exposure and only few data on the endogenous production of amines, it is likely that sufficient quantities of amines are present in humans to participate in nitrosation reactions under appropriate conditions. It is also probable that certain environments, as well as exposure to tobacco and certain drugs, will result in increased exposure to these compounds. A sufficient body of evidence demonstrates that amines derived from both exogenous and endogenous sources could participate in in vivo nitrosation reactions to produce nitrosamines, many of which are carcinogenic.

AGENTS ENHANCING OR INHIBITING IN VIVO NITROSATION

The formation of N-nitroso compounds can be increased, decreased, or even completely blocked by the presence of certain agents in the reaction mixture (Douglass et al., 1978) (see Chapter 4). The ability of many chemicals to catalyze and inhibit nitrosation has been demonstrated in simple chemical systems (Boyland et al., 1971; Mirvish et al., 1972), in model food systems (Gray and Dugan, 1975; Massey et al., 1978), and in simulated gastric or salivary contents (Tannenbaum et al., 1977; Ziebarth and Scheunig, 1976). Nitrosation reactions have also been demonstrated in in vivo systems for example, in the

TABLE 6-3

Endogenous Amines

Amine	Structure	Source	Reference
Betaine	$(\text{CH}_3)_3\text{N}^+-\text{CH}_2-\text{COO}^-$	Widely distributed in plants and animals	Fiddler <u>et al.</u> 1972
Cadaverine	$\text{H}_2\text{N}-(\text{CH}_2)_5-\text{NH}_2$	Bacterial decarboxylation of lysine	Johnson, 1977
Carnitine	$(\text{CH}_3)_3\text{N}^+-\text{CH}_2-\underset{\text{OH}}{\underset{ }{\text{CH}}}-\text{CH}_2-\text{COO}^-$	Constituent of striated muscle and liver; meat	Fiddler <u>et al.</u> 1972
Choline	$(\text{CH}_3)_3\text{N}^+-\underset{\text{OH}}{\underset{ }{\text{CH}}}_2\text{CH}_2\text{OH}$	Metabolism of lecithin by bacterial phospholipases; can also be dealkylated to form dimethylamine	Johnson, 1977
N,N-Dimethylglycine	$\begin{array}{c} \text{CH}_3 \backslash \\ \text{N}-\text{CH}_2-\text{COOH} \\ \text{CH}_3 / \end{array}$	Unknown	Friedman and McClanahan, 1973; Mirvis 1975
Neurine chloride	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_2=\text{CH}-\text{N}^+-\text{CH}_3 \\ \\ \text{CH}_3 \end{array} \text{Cl}^-$	Egg yolk, bile in cadavers	Fiddler <u>et al.</u> 1972
Piperidine		Metabolism of lysine by gut flora	Hawksworth and Hill, 1971; Hill, 1979; Johnson, 1977
Putrescine	$\text{H}_2\text{N}-(\text{CH}_2)_4-\text{NH}_2$	Bacterial decarboxylation of ornithine	Johnson, 1977
Pyrrolidine		Metabolism of ornithine and arginine by gut flora	Johnson, 1977

1973, Narviss et al., 1975) and in biological materials that were analyzed for the presence of N-nitroso compounds (Kamm et al., 1975).

The assessment of the significance of catalysts and inhibitors for the in vivo formation of N-nitroso compounds requires a discussion of the environmental distribution and possible exposure of humans to both catalysts and inhibitors. The catalysts that are considered in this section are thiocyanate, halide ions, and certain phenols. The inhibitors reviewed herein include ascorbic acid, bisulfite ion, and several phenols and thiols. Not all modifying agents of possible significance in vivo are included; rather, the discussions concern agents that illustrate some of the complexities involved in predicting both qualitative and quantitative outcome of nitrosation reactions. Although information on possible exposures of humans may indicate which chemicals could be important in vivo, the absolute intake of any one substance is less meaningful in predicting catalysis or inhibition than is the intake of these modifying agents relative to the intake of nitrate, nitrite, and nitrosatable substrates.

Other important factors that determine the effect of catalysts and inhibitors are the timing of intake (relative to the intake of nitrate, nitrite, and amino substrates), the persistence of the various substances in vivo, and the reactivity of the modifying agents. For example, approximately 25% of ingested nitrate is recycled in the bloodstream to the mouth where some of it is reduced to nitrite. This contribution of salivary nitrite to the total nitrite content of the stomach will continue over time. Thus, in order to be effective, inhibitors of nitrosation should either be persistent or administered frequently.

In addition, both the pH and the nature of the medium (i.e., hydrophilic or lipophilic) influence the outcome of nitrosation reactions in the presence of modifying agents. Much of the information about the mediatory effects of various compounds has been obtained from investigations with model systems that have used relatively high concentrations of single compounds in homogeneous media. Thus, the extrapolation of these findings to the heterogeneous conditions that may exist in vivo may be an oversimplification and may lead to the possibility of synergistic or interfering effects being overlooked.

Catalysts

Several nucleophilic anions, especially thiocyanate and iodide, may catalyze the nitrosation reactions of nitrite under acidic conditions in the stomach, but not at the neutral pH of normal saliva.

intestinal tract.

Of the anionic catalysts studied thus far, thiocyanate has been found to have the greatest effect (Boyland, 1972; Boyland et al., 1971; Fan and Tannenbaum, 1973; Mirvish, 1975). In one study of factors influencing the rate at which morpholine is nitrosated, Fan and Tannenbaum (1973) found that the optimal pH for catalysis by thiocyanate was 2.3 and that catalysis did not occur as readily above pH 3 and below pH 2.

Both the dependency on pH and the intensity of thiocyanate catalysis are strongly influenced by the structure of the amino substrate. Thus, no pH maximum is observed for the nitrosation of weakly basic amines such as N-methylaniline, where catalysis by thiocyanate is strong (Boyland et al., 1971). By contrast, thiocyanate does not catalyze the nitrosation of ureas, amides, guanidines, and urethanes (see Chapter 4).

Halide ions are also catalytic. Iodide is more reactive than bromide, which is more reactive than chloride (Boyland, 1972; Fan and Tannenbaum, 1973; Schweinsberg, 1975). The effect of pH on these compounds is very similar to that described for thiocyanate (Fan and Tannenbaum, 1973).

Nitrosation by the thiocyanate-NO and halide-NO reagents should be more important for low rather than high nitrite concentrations because the concentration of N_2O_3 (which regulates noncatalyzed nitrosation of secondary amines) depends on the square of the concentration of nitrite, whereas the concentration of the nitrosating agents in the presence of thiocyanate and halide ions ($SCN-NO$ and $X-NO$) depends simply on the level of nitrite. As a consequence, the levels of nitrosamines formed in vivo, when the concentration of nitrite is low, may be underestimated if the reaction is assumed to occur via N_2O_3 and if the presence of catalytic anions is not taken into consideration.

All phenolic compounds can combine irreversibly with nitrosating agents to form nitroso products, which can catalyze the formation of nitrosamines from nitrous acid under acidic conditions (see Chapter 4). Phenols that can act as catalysts following their nitrosation are monohydroxy compounds without 2-, 4-, or 6-substituents and 1,3-dihydroxy compounds. Thus, certain phenols, including a group of naturally occurring compounds (e.g., catechin, quercetin, and kaempferol) tested by Pignatelli et al. (1980), can catalyze the formation of nitrosamine by nitrous acid, when the concentration of nitrous acid exceeds that

thiocyanate is present in the saliva of humans at concentrations ranging from 11.7 to 33 mg/100 ml (Diem, 1962); in the saliva of smokers, concentrations of this compound are nearly 3 to 4 times higher (Boyland and Walker, 1975; Druckrey et al., 1967; Schievelbein et al., 1969). The anion is also present in gastric secretions at 1 mg/100 ml (Lane and Bailey, 1973).

Varying amounts of iodide occur in food, water, medications, and air (Underwood, 1977). The most important sources include iodized salt, bread, milk, marine fish, and other seafood (Kidd et al., 1974). Average dietary intake ranges from 0.24 to 0.74 mg/day (Oddie et al., 1970).

Bromide is found in certain medications and brominated vegetable oils. The average daily intake has not been estimated.

The chloride ion has been shown to catalyze the diazotization of aromatic amines, which proceeds via formation of the primary nitrosamine (Ridd, 1961), but it has never been shown to catalyze the formation of N-nitroso compounds. The occurrence of chloride is briefly reviewed below in case future studies demonstrate that it does have an effect on nitrosation.

Chloride is found primarily in food (mainly as sodium chloride) and water. Rich dietary sources include table salt, breakfast cereals, breads, dried skim milk, teas, eggs, margarine, salted butter, bacon, ham, salted beef, canned meats, canned fish, canned vegetables, salted snack foods, and olives (Harper et al., 1977). Dietary levels of chloride depend largely on the intake of table salt. Sodium chloride alone is estimated to provide from 2,400 to 14,400 mg/day (Dahl, 1960). The EPA has estimated that the average chloride content of drinking water is 21 mg/liter (U.S. Environmental Protection Agency, 1975). Assuming that 2 liters of water are consumed daily, this source may contribute 42 mg/day or just less than 2% of the lower estimate for the daily intake from sodium chloride.

Many of the phenols that are capable of catalyzing nitrosation reactions are found in plants and, hence, in food prepared from these plants. The average daily intake of phenols in the diet has not been determined; however, the total phenolic content of certain fruits and vegetables may be quite high. For example, the phenolic content of unripe persimmons is 3 g/kg (Nakayama and Chichester, 1963). Coffee and tea may contribute 1 g to the total intake of phenols per day (Anonymous, 1969). However, when considering the intake of phenols from fruits and vegetables, one should remember

Inhibitors

In general, the most effective inhibitors of nitrosation reactions act by rapidly reducing the nitrosating agent to either nitric oxide or nitrogen. Ascorbic acid (vitamin C) blocks the formation of nitrosamines in a number of systems (Archer et al., 1975; Bruce et al., 1979; Ivankovic et al., 1975; Kamm et al., 1975; Mirvish et al., 1972). This agent is most effective at pH 1 to 4 in the absence of oxygen when its concentration is at least equal to that of nitrite (Archer et al., 1975; Mirvish, 1981a). As expected, the amines that are rapidly nitrosated are less susceptible to inhibition than are the ones that are nitrosated more slowly (Mirvish, 1981b). Because vitamin C is water soluble, it is less effective in lipophilic media (Pensabene et al., 1976).

α -Tocopherol (vitamin E) is also capable of blocking nitrosation reactions. It can be as effective as ascorbic acid, but only in lipophilic media or in emulsions (Kamm et al., 1977; Mergens et al., 1978; Tannenbaum and Mergens, 1980; Walters et al., 1976). Again, the relative concentrations of the inhibitor and nitrite are important in determining the outcome. The optimum pH range for the reaction is 2 to 3. As the pH is increased, the reaction between α -tocopherol and nitrite slows down until the pH reaches 5, when less than 5% of the nitrite reacts with this agent (Mergens et al., 1978); however, the formation of nitrosamines also decreases with increasing pH.

Certain phenols can also inhibit the formation of nitroso compounds by competing with the amino substrates and combining irreversibly with the nitrosating species. In the presence of excess nitrosating agent, however, subsequent interactions between the nitrosated phenol and the nitrosating agent may then catalyze the formation of nitrosamines (see above). Other phenols, which contain two hydroxyl groups in the 1,2 or 1,4 positions, inhibit the formation of nitroso compounds by reducing the nitrosating agent to nitric oxide. In principle, these phenols should be as effective as ascorbic acid as blocking agents when used at pH 1 to 4 under anaerobic conditions and when the concentration of phenol is at least equal to that of nitrite.

Sodium bisulfite has also been extensively tested. At a strongly acidic pH, the effectiveness of this compound has been found to equal that of ascorbic acid and α -tocopherol (Mirvish, 1975). At pH 1 to 4, bisulfite reduces nitrite in two steps: first to nitric oxide and then to nitrous oxide (Hisatsune, 1961).

promote rather than inhibit the formation of nitrosamines under such conditions.

Environmental Distribution and Exposure of Humans to Inhibitors

Ascorbic acid is present in a variety of fresh fruits and vegetables in a wide range of concentrations. For example, oranges contain 50 mg/100 g, whereas apples contain 4 mg/100 g (U.S. Department of Agriculture, 1963). The concentration in fresh foods may be reduced considerably during storage or cooking. Ascorbic acid or ascorbate is also added to many food items, including baked goods, cereals, milk, frozen dairy products, meat (including cured meats), poultry, fish, processed vegetables, jam, soups, nonalcoholic beverages, beer, candy, and infant formulas.

The U.S. Department of Agriculture (1980) has estimated that the average daily dietary intake of vitamin C is 87 mg/person. However, a committee of the National Academy of Sciences (1973) estimated that the daily intake of ascorbic acid added to food could be as high as 550 mg. Not included in either of these figures is the contribution of vitamin supplements. According to unpublished data from the same USDA survey (personal communication), approximately 24% of all Americans surveyed were taking some form of vitamin supplementation. An additional 7% were specifically taking vitamin C supplements; however, the quantities were not reported.

α -Tocopherol is a natural constituent of certain foods. Fresh pork bellies may contain up to 20 mg/kg, which declines to less than 5 mg/kg upon processing into bacon. Other dietary sources include milk products, poultry, gelatin, soups, breakfast cereals, and infant formulas. Current estimates of daily intake of α -tocopherol derived from foods range from 3.8 to 11.8 mg/day (U.S. Department of Agriculture personal communication). However, if concentrations of α -tocopherol added to foods are also considered, an estimated 54.9 mg may be consumed per person per day (National Academy of Sciences, 1973). α -Tocopherol may also be present in multiple vitamin formulations taken by 24% of the U.S. population, and it is taken by itself as a supplement by 4% of those surveyed by the USDA (personal communication). However, the unesterified form of α -tocopherol is required to block nitrosamine formation. That form of the vitamin occurs naturally in foods. Most vitamin supplements contain the acetate ester form of the vitamin which does not act as a blocking agent (Tannenbaum and Mergens, 1980).

Tannins (tannic acid), propyl gallate, vanillin, chlorogenic acid, and thymol are phenols that may have an inhibitory effect on the formation of N-nitroso compounds. Tannic acid is a naturally occurring component of foods prepared from plants. The total intake of tannins may be as high as 1 g per day for persons who drink coffee and tea (Singleton and Kratzer, 1973). Chlorogenic acid is found in coffee and in many plant materials. The possible exposure of humans to phenols that are added to foods may be 3.9 mg/day for propyl gallate; up to 500 mg/day for vanillin (if all forms of vanilla are added together); and 3.5 mg for thymol (National Academy of Sciences, 1973). Concentrations of phenols as high as 300 mg/kg have also been found in smoked meats (Knowles et al., 1975).

Sodium bisulfite is a food additive. It is used in baked goods, processed fruits and vegetables, beverages, and relishes. Daily intake has been estimated to be 187.2 mg/day (National Academy of Sciences, 1973).

Free thiol groups, equivalent to 21-25 mM, have been found in meat (Hamm and Hofmann, 1966).

The amounts of inhibitors ingested by humans are not as important as the ratio of inhibitors to nitrite in the stomach. Thus, determining the level of nitrite present in human gastric juice is an important first step in determining the concentration of the inhibitor that is required (Ruddell et al., 1977) (see Chapter 8). In an investigation conducted in humans, Ohshima and Bartsch (1981) studied the amount of inhibitors required to block N-nitroso compound formation. These investigators showed that the formation of nitrosoproline following ingestion of proline and red beet juice was completely inhibited by a large excess of ascorbic acid and partially blocked by an excess of α -tocopherol. The implication of this finding for predicting the amount of inhibitors generally needed to block nitrosation in the human stomach, where most in vivo nitrosation is likely to occur, remains unknown.

In addition, the persistence of the inhibitor in the stomach is also an important factor since nitrite may be continually introduced into the stomach from swallowed saliva. Investigators have found that tissue levels of α -tocopherol can be raised in many organs of rats by supplementing their diet with the vitamin (Mergens et al., 1978). The persistence of α -tocopherol in certain organs, such as the stomach and lung, may make this inhibitor of nitrosation reactions of greater importance than inhibitors that do not persist as long.

it is impossible to predict the outcome of reactions within such mixtures solely on the basis of the estimated intakes of catalysts and inhibitors, even when such data are compared to intakes of the reactants. Hence, although it seems likely that modifying agents play a role in in vivo nitrosation reactions, the eventual outcome cannot be predicted with any degree of precision.

Although one study in humans has demonstrated that the nitrosation of proline was inhibited by ascorbic acid and α -tocopherol, the amount of inhibitors required for this blocking effect remains unknown. Determining the nitrite content of gastric juice at various times would be an important first step in ascertaining the level of inhibitors needed.

Decreasing the intake of catalysts or increasing the intake of inhibitors (if it could be easily accomplished) may not necessarily reduce the in vivo formation of N-nitroso compounds. First, the adjustment must be timed to coincide with the consumption of nitrite. Moreover, some compounds, such as certain phenols and thiols, can act either as catalysts or inhibitors, depending on the reaction conditions. Another complication is that many dietary sources of ascorbic acid also contain nitrate (see Chapter 5). Thus, the effect on in vivo nitrosation from such dietary sources will depend on the ratio of ascorbate to nitrate. Moreover, an increased intake of inhibitors may be unwise if adequate toxicological information is not available or if the agent is clearly toxic. The elimination of catalysts must also be viewed with caution because they may be essential constituents of the diet.

More information on the in vivo effects of the various agents is needed because some modifiers may be of greater significance than others, depending on their reactivity and/or the extent to which humans are exposed to them. Persistence is another important consideration. For example, α -tocopherol has been shown to persist in various organs such as the lung and stomach of laboratory animals.

OVERALL SUMMARY AND CONCLUSIONS

The formation of N-nitroso compounds in vivo is dependent on the relative concentrations of nitrate, nitrite, nitrosatable substances, and agents that can either enhance or inhibit nitrosation reactions.

Nitrosatable substances, especially amines, are present throughout the environment and may be produced endogenously as well. Although

settings, in smokers, and in individuals taking certain drugs.

In addition to the amounts and types of nitrosating species and substrates, there are many other factors that influence the nitrosation reaction. Thus, it is difficult to predict the extent to which N-nitroso compounds will be formed endogenously from a given mixture of reactants. One important consideration is the presence of modifiers (catalysts and inhibitors) of the nitrosation reaction. Compounds such as thiocyanate, iodide, and bromide, can catalyze nitrosation, whereas ascorbic acid, α -tocopherol, and thiols can inhibit the reaction. Some compounds, such as phenols, can either inhibit or catalyze, depending on the conditions. The presence of many other catalysts and inhibitors not discussed in this chapter may also influence the amount of N-nitroso compounds formed endogenously.

RECOMMENDATIONS

In certain circumstances, some subgroups of the population may be exposed to excessive levels of nitrosatable amines or amides. The committee recommends that such exposures be reduced, when feasible. For example, pesticides produced as secondary and tertiary amine salts could be substituted by other formulations, and certain readily nitrosatable drugs could be replaced by drugs that have the same therapeutic effect but are not nitrosatable. In addition, further research should be performed to identify amino compounds that could be nitrosated in vivo--especially those that are readily nitrosated or to which there is a large exposure.

The committee recommends that further research be conducted to study inhibition and catalysis of nitrosation reactions in vivo, specifically to determine the amount of nitrite that is destroyed in the human stomach and the extent to which nitrosation reactions are modified by the various inhibitors. Attention should also be directed toward interactions among inhibitors, catalysts, and other food-derived substances.

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CHAPTER 7

N-NITROSO COMPOUNDS: ENVIRONMENTAL DISTRIBUTION AND EXPOSURE OF HUMANS

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N-NITROSO COMPOUNDS: ENVIRONMENTAL DISTRIBUTION AND EXPOSURE OF HUMANS

Several analytical methods have been used to determine the levels of volatile nitrosamines in a variety of environmental media. In this chapter, the committee has described these methods and their limitations and has reviewed the resultant data on nitrosamine concentrations in tobacco, food, alcoholic beverages, cosmetics, pharmaceuticals, pesticides, water, the atmosphere, and occupational settings. Estimates of individual exogenous exposures to nitrosamines for workers in certain occupations, for tobacco users, and for the general population of several countries, based on concentrations of nitrosamines in food and alcoholic beverages are also presented. In addition, the committee has used the concentrations of nitrosamines in various environmental sources to develop estimates of average exposures to nitrosamines for the U.S. population.

ANALYTICAL METHODS

Methods that have withstood the test of extensive interlaboratory collaborative studies exist only for N-nitroso compounds that are amenable to analysis by gas chromatography (GC). Procedures for N-nitroso compounds that decompose upon heating or are not sufficiently volatile to be analyzed by GC are more complex. Only a few compounds with such properties have been analyzed by high pressure liquid chromatography (HPLC) with a Thermal Energy Analyzer (TEA®) or an ultraviolet detector. Recently, a method for the detection of nitrosamides has been reported (Saul et al., in press).

Techniques used for the analysis of volatile and nonvolatile N-nitroso compounds include thin-layer chromatography, polarography, spectrophotometry, and chemical denitrosation reactions. Recently, the TEA and the high-resolution mass spectrometer, when used as detectors for gas chromatographs, have become the instruments of choice for volatile N-nitroso compounds (International Agency for Research on Cancer, 1978a,b, 1980).

Gough et al. (1977c) compared the GC-TEA procedure for food and urine samples with three GC-mass spectrometric (GC-MS) techniques. Low-resolution MS was performed by monitoring two key ions

comparing the peak to that of a suitable standard. The investigators demonstrated that only the high-resolution MS with peak matching provided data that were in agreement with those obtained with the TEA. For example, a urine sample was found to contain more than 100 $\mu\text{g/liter}$ (ppb) nitrosodimethylamine (NDMA) by low-resolution MS in which two peaks were monitored, 15 $\mu\text{g/liter}$ by high-resolution MS with precise ion monitoring, but only 2 $\mu\text{g/liter}$ by high-resolution MS with peak matching. The TEA result was 1.2 $\mu\text{g/liter}$. In a sample of Chinese food, low-resolution MS detected nitrosopyrrolidine (NPYR) at a concentration of 70 $\mu\text{g/kg}$, whereas high-resolution MS with precise ion monitoring detected NPYR at 3 $\mu\text{g/kg}$. Yet, NPYR was not detectable either by high-resolution MS with peak matching or by TEA. In this case, not only was the quantitation in error, but an inappropriate use of MS led to a positive result when no nitrosamine was present. The work of Gough and his colleagues clearly demonstrates the need to ensure that the MS is carried out with appropriate safeguards.

Havery et al. (1978) found no discrepancies when comparing 106 cured meat samples by GC-TEA and GC-MS. Webb et al. (1979) demonstrated good agreement, even at levels less than 1 pg, when the extracts of 98 substrates were compared on GC-TEA and GC-MS. These papers demonstrate that GC-TEA and GC-MS (high resolution with peak matching) can be used for the reliable identification of trace levels of volatile nitrosamines extracted from complex matrices.

Although both the TEA analyzer and the high-resolution spectrometer have been shown to be reliable tools for identifying nitrosamines in extracts, even at the picogram level, it is still difficult to ensure that the nitrosamines detected were present in the sample before extraction. Artifacts arise because precursors of nitrosamines are generally present in much larger amounts than the nitrosamines themselves. Furthermore, depending upon the matrix and precursors, the formation of artifacts can be enhanced, or the nitrosamine destroyed, by the action of acid, alkali, heat, light, radiation, and other factors. Artifactual formation presents especially acute problems when attempting to detect nitrosamines in biological fluids where levels are likely to be less than 1 $\mu\text{g/liter}$. The control of artifacts during analysis for nitrosamines has been discussed in detail by Krull et al. (1978, 1979a). In general, many investigators now agree that whenever an N-nitroso compound is first reported to be present in a new sample matrix, the data should be presumed to indicate that artifacts have been formed during analysis, unless all or most of the following specific steps have been taken:

- Glassware, solvents, and other testing equipment and materials have been checked daily for contamination.

- The minimum possible number of analytical steps have been used. If possible, at least one data point is checked by introducing the crude sample directly into a GC-TEA (Fan et al., 1977b). Even then, care must be exercised to prevent formation of artifacts in the GC injector itself (Fan and Fine, 1978).

- Consideration has been given to the fact that many solvents (Eisenbrand et al., 1978), amines, deionized water (Kimoto et al., 1980), and all rubber products are contaminated with nitrosamines.

- Undue exposure to ambient air is avoided since nitrogen oxides, even at ambient levels, can nitrosate amines upon contact (Eisenbrand et al., 1978). (See Chapter 4.)

Specific analytical procedures for nonvolatile nitroso compounds such as the N-nitroso ureas, amides, and carbamates have only recently been developed. For example, a colorimetric method for urea can be used to determine nitrosoureas and nitrosocyanamides (Mirvish et al. 1979), and Saul et al. (in press) have recently reported a method for detecting nitrosamides. There are also some broad screening techniques that can be used for some nonvolatile N-nitroso compounds (Fan et al., 1978a; Fine, 1980b). For example, screening techniques have been used successfully to identify a variety of nonvolatile nitrosamines in pesticide products (Wolf et al., 1980; Zweig and Garner, in press). Final detection has generally been made by HPLC-UV (ultraviolet) or HPLC-TEA techniques. In tobacco and tobacco smoke, the presence of tobacco-specific nonvolatile nitrosamines has been detected by HPLC-TEA and other HPLC procedures (Hecht et al., 1978; Hoffmann et al., 1979).

Procedures have been developed to analyze foodstuffs for such compounds as the nitrosamino acids. Nitroso-3-hydroxypyrrolidine, which was found in cured meat products (Janzowski et al., 1978), was determined by trifluoroacetylation, followed by GC-TEA and GC-high-resolution MS. Sen et al. (1977a) have also described a mass spectrometric method to detect this compound in cooked bacon. More recently, Roussin's red methyl ester $[(NO)_2Fe(CH_3S)]_2$ was isolated and identified by GC-MS from the ether extracts of Chinese vegetables (Lu et al., 1981). The total N-nitroso compound content of food samples has been assayed by Walters et al. (1978), who used a chemical denitrosation procedure, followed by the detection of the nitrosyl radical by its chemiluminescence reaction with

important because of the potential of these agents to form N-nitroso compounds via transnitrosation reactions (see Chapter 4). These nitroso compounds are generally thermolabile and readily decompose to release nitric oxide (Krull et al., 1979a). Specific analytical procedures have not yet been identified, although some authors have reported that these compounds can be detected with the TEA. Krull et al. (1979a) have described procedures for distinguishing these compounds from N-nitroso compounds.

CONCENTRATIONS OF NITROSAMINES IN VARIOUS ENVIRONMENTAL SOURCES

Occupational Settings

The highest known concentrations of exogenous nitrosamines occur in the workplace, especially in the rubber and leather-tanning industries.

Rubber Industry. The rubber industry uses nitrosodiphenylamine (NDPhA) as a vulcanization retarder. This nitrosamine has been shown to be carcinogenic in rats (Cardy et al., 1979). It is very labile, can participate in transnitrosation reactions (see Chapters 4 and 6), and may contribute to the formation of other carcinogenic N-nitroso compounds.

Fajen et al. (1979) measured levels of nitrosamines in three rubber factories. As expected, NDPhA was found at the 0.2 to 47 $\mu\text{g}/\text{m}^3$ level in the air of a factory where the compound was being manufactured. The entire area was contaminated with NDPhA; its concentration in a mud scraping from the floor contained 15,000 mg/kg. In addition to the presence of NDPhA in chemical manufacturing areas, curing and extrusion sections of the rubber factories were found to contain nitrosomorpholine (NMOR) in concentrations ranging from 0.5 to 27 $\mu\text{g}/\text{m}^3$. (The NMOR presumably arises from the use of bismorpholinecarbamylsulfenamide, which is used as an accelerator.) NDMA was detected at lower levels (0.05 to 0.5 $\mu\text{g}/\text{m}^3$) as an air pollutant in several of the factories.

More recently, McGlothlin et al. (1981) conducted an in-depth study of nitrosamine levels in a rubber factory. Initial measurements indicated that one area air sample contained NMOR at 250 $\mu\text{g}/\text{m}^3$. Within 7 months, airborne nitrosamine levels were reduced dramatically (McGlothlin et al., 1981). As shown in Figure 7-1, 250 $\mu\text{g}/\text{m}^3$ was the highest atmospheric concentration of NMOR found at the beginning of the study in August 1979. Within 2 months, the concentration had been reduced to 120 $\mu\text{g}/\text{m}^3$ by venting the building. Two months later,

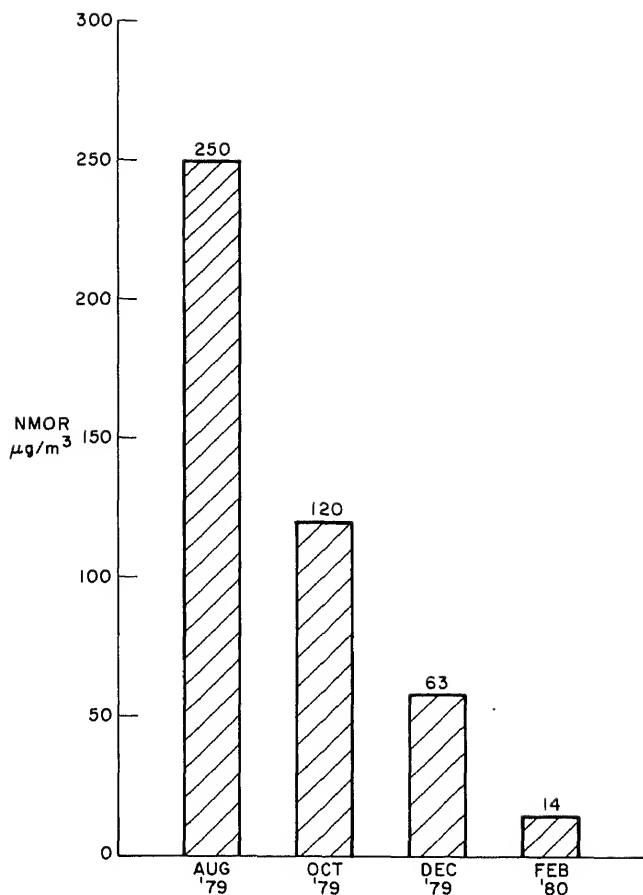


FIGURE 7-1. Highest NMOR concentrations in area air samples obtained from a rubber factory between August 1979 and February 1980, showing reductions achieved. The October 1979 reduction was attributed to venting, the December 1979 reduction to the installation of exhaust canopies, and the February 1980 reduction to the use of a phthalimide derivative instead of NDPhA. From Fine and Rounbehler, in press.

Preussmann et al. (1980; in press) measured nitrosamine levels in 19 rubber factories in the Federal Republic of Germany. Initial concentrations of NDMA and NMOR ranged from 1 to 20 $\mu\text{g}/\text{m}^3$. In one working area (tire tube curing), an extremely high concentration of NDMA (140 $\mu\text{g}/\text{m}^3$) was detected. Subsequently, levels in that area were reduced 1 to 2 $\mu\text{g}/\text{m}^3$ by substituting the NDPhA with cyclohexylthiophthalimide. More recently, Spiegelhalder and Preussmann (in press) have reported that air in certain areas of rubber factories in Germany may contain extremely high concentrations of nitrosamines--1,060 $\mu\text{g}/\text{m}^3$ NDMA and 4,700 $\mu\text{g}/\text{m}^3$ NMOR.

Leather Tanning. Because of Acheson's (1976) report of a possible increase in nasal cancer incidence among leather workers, an investigation of airborne nitrosamine levels in a typical leather tannery was undertaken. In their initial study, Rounbehler et al. (1979) reported that NDMA was detected as an airborne pollutant at all sites in the tannery on three separate visits. The highest level, 47 $\mu\text{g}/\text{m}^3$, was found in the retanning, coloring, and fat-liquoring areas. The average NDMA level on the first and second visits was 19 $\mu\text{g}/\text{m}^3$. Small amounts of NDMA were also found in the process water and wastewater. By the time of the third visit, the tannery had been thoroughly cleaned and NDMA levels had been reduced to a range of 0.1 to 3.4 $\mu\text{g}/\text{m}^3$.

This group has now surveyed eight leather-tanning facilities throughout the United States (Fajen et al., in press; New England Institute for Life Sciences, in press). Four of the eight plants were found to have airborne concentrations of NDMA greater than 0.5 $\mu\text{g}/\text{m}^3$. Table 7-1 lists the type of operations of each plant and the highest levels of NDMA found at that plant. The data in the table indicate that the use of dimethylamine sulfate (DMAS), a precursor of NDMA, as a depilatory agent, is associated with the presence of airborne NDMA. Even a facility that had recently discontinued the use of DMAS, and another that used DMAS only on an experimental basis, contained airborne NDMA.

The agents nitrosating the DMAS are probably oxides of nitrogen formed by the combustion of fossil fuels in gas-powered forklift trucks or in open gas heaters; however, because all the tanneries that used DMAS had a possible source of nitrogen oxides, this could not be determined conclusively.

In further studies aimed at determining the source of NDMA, airborne nitrosation potential and levels of amines and nitrosamines were measured simultaneously throughout a complete tanning operation

(Fine and Rounbehler, in press). None of the bulk samples that were collected contained NDMA (sensitivity limit 0.5 µg/ml), including the fresh DMAS, the hide depilatory solution, and even water on the floor near the depilatory operation. This negative finding demonstrates conclusively that the source of NDMA is not an impurity in the DMAS, nor is NDMA being formed in the depilatory solutions. However, NDMA was present in the air at all test sites in the tannery at the time the bulk samples were collected. The data on airborne concentrations also show that dimethylamine (DMA) was always present (Table 7-2). In all cases, the amount of airborne NDMA was approximately equal to 1% of the amount of airborne DMA. In contrast, NDMA levels did not vary with measured nitrosation capacity presumably because nitrosation capacity (i.e., nitrogen oxide levels) did not vary sufficiently among the different areas in the tannery.

As shown in Table 7-2, DMA, presumably formed from the DMAS, is clearly needed to produce detectable levels of NDMA. Yet, as was shown in a previous study, the DMAS itself and its aqueous solutions do not contain NDMA. A much higher level of DMA, as well as sufficient airborne nitrosation capacity, is required for the production of airborne NDMA. Thus, the NDMA must be formed from DMA outside the solution -- either in the gas phase or on surfaces.

TABLE 7-1

Summary of NDMA Concentrations Measured at Eight
Leather-Tanning Facilities^a

Description of Tannery ^b	DMAS Used	Source of Nitrogen Oxides	Highest NDMA Concentration Measured, µg/m ³
All operations	Yes	Fork-lift trucks	47
All operations	Yes	Fork-lift trucks	11
All operations	No	Fork-lift trucks	0
All operations	No	Fork-lift trucks	0
Partial-wet	Recently discontinued	Fork-lift trucks	8
Partial-wet	Used experimentally	Open gas heaters	3
Partial-dry	No	Fork-lift trucks	0.05
Partial-dry	No	None	0

NOTE: Some concentrations have been rounded off
to two significant figures.

<u>Sample^b</u>	<u>Nitrogen Oxides, ppb^c</u>	<u>DMA, $\mu\text{g}/\text{m}^3$</u>	<u>NDMA, $\mu\text{g}/\text{m}^3$</u>	<u>NDMA/DMA, %</u>
1	58	490	4.6	0.9
2	37	280	5	1.8
3	67	180	1.2	0.6
4	88	260	3.3	1.3
5	80	280	3.6	1.3

^aFrom Fine and Rounbehler, in press; each concentration given is the mean of three samples taken on three different days.

^bSamples were taken at various locations within the plant.

^cNitrogen oxides were measured indirectly as airborne nitrosation potential (see text).

Amine Factories. Bretschneider and Matz (1973, 1976) reported the presence of trace levels of NDMA in the air of a factory producing "fat" chemicals and one manufacturing pharmaceuticals. They also reported levels between 1 and 43 $\mu\text{g}/\text{m}^3$ on the site of a plant manufacturing DMA. Fine et al. (1976b) reported NDMA concentrations ranging from 0.01 to 1 $\mu\text{g}/\text{m}^3$ in the ambient air outside a factory in Belle, W. Va., in which DMA was manufactured and used. Subsequent studies (Fine et al., 1977a) showed that the source of the NDMA was a vent from a pilot chemical manufacturing operation (levels up to 130 $\mu\text{g}/\text{m}^3$). The NDMA was apparently produced as an unwanted by-product. Atmospheric concentrations of NDMA in the neighboring towns of Belle and Charleston ranged from 0.001 to 0.04 $\mu\text{g}/\text{m}^3$. Apparently, most of the NDMA detected had been produced in the chemical plant and not by the reaction of DMA with oxides of nitrogen in the atmosphere (Fine et al., 1977a).

Rocket Fuel Factory. Fine et al. (1976a,c) reported that NDMA was present as an air pollutant in Baltimore, Md. The prime source

approximately 1 µg/m³ was measured in the residential neighborhood adjacent to the factory, and approximately 0.1 µg/m³ was found approximately 3.2 km away in downtown Baltimore (Fine et al., 1976a,b, 1977a,b,c).

A leak of NDMA as small as 130 g (4.7 oz) per hour could have been responsible for all of the airborne concentrations of NDMA found in Baltimore. The UDMH factory had been in operation for 17 years (from 1956 to 1973) before it was rebuilt in 1973 as a sealed system to comply with safety guidelines issued at that time by the Occupational Safety and Health Administration (OSHA) for handling carcinogens such as NDMA. Unfortunately, no data on airborne NDMA from that source were available prior to August 1975, and this factory ceased manufacturing rocket fuel in 1976.

Machine Shops. The finding of nitrosodiethanolamine (NDELA) in some industrial fluids at the 3% level (Fan et al., 1977b) triggered an effort to determine the extent of worker exposure to airborne NDELA. Fortunately, NDELA was not detected as an airborne pollutant in factories making the cutting fluids or in large and small machine shops using the fluids (New England Institute for Life Sciences, in press).

Despite the fact that NDELA is not sufficiently volatile to pose a problem as an air pollutant, significant exposure of workers can still occur by dermal contact, either through splashing or by handling metal parts that have been soaked in the fluids. Concern for these workers has increased as a result of recent reports that NDELA penetrates the skin of both rats (Lijinsky et al., 1981) and humans (Bronaugh et al., 1981; Edwards et al., 1979) and that it is a more potent carcinogen than was previously surmised (Lijinsky et al., 1980; Preussmann et al., in press a). NDELA is also present in cosmetics. This is discussed in a later section of the chapter.

Tobacco and Tobacco Smoke

At the time of harvesting, fresh tobacco leaves do not contain measurable amounts of nitrosamines (< 5 µg/kg). However, these compounds are formed during curing, aging, and fermentation. Their concentrations depend primarily on the amount of nitrosatable precursors -- proteins, alkaloids, agricultural chemicals -- and the amount of nitrate present in the fresh tobacco as well as on the processing conditions, which lead to the reduction of the nitrate to nitrite. The volatile nitrosamines NDMA and nitrosodiethylamine (NDEA) have been detected in various tobacco products (Table 7-3); however, the amounts detected are much less than those for pesticide-derived and

NOTE: Some concentrations have been rounded off to two significant figures.

<u>Tobacco</u>	<u>Concentrations, $\mu\text{g/kg}$</u>	
	<u>NDMA</u>	<u>NDEA</u>
Cigar (Pennsylvania) ^b	6.9	NR ^c
Robinson, high nitrate ^d	9.5	15
Catterton, high nitrate ^d	16	12
French cigarette	188	12
Fine-cut chewing tobacco	56	8.6

^aAdapted from Brunneemann et al., 1977.

^bCommercial product bought in open market.

^cNo data reported.

^dTobacco from experimental cigarettes with high nitrate levels provided by the U.S. Department of Agriculture.

TABLE 7-4

Nitrosamines in Snuff and Chewing Tobacco^a

NOTE: Some concentrations have been rounded off to two significant figures.

<u>Tobacco Product</u>	<u>NDELA, $\mu\text{g/kg}$</u>	<u>Tobacco-Specific Nitrosamines, mg/kg</u>			
		<u>NAT</u>	<u>NNN</u>	<u>NNK</u>	<u>Total</u>
Snuff, fresh	6,800	-	-	-	-
Snuff, aged	3,200	-	-	-	-
U.S. snuff	-	2-44	3.5-39	1.3-4.6	6.8-88
Bavarian snuff	-	4	6	1.5	12
Chewing tobacco	220-280	-	-	-	-

believed to be derived from diethanolamine (DELA), which is present in maleic hydrazide-diethanolamine (MH-30), a pesticide used in the cultivation of tobacco. Pesticide formulations also contain numerous other nitrosatable amines (Chapter 6), which could give rise to nitrosamines not only in the pesticides themselves, but also in the tobacco to which they are applied. However, despite the fact that residues of such amine-containing pesticides as methylcarbamate and carbaryl (Sevin®) have been detected in harvested tobacco (Sheets and Leidy, 1979), there have been no studies of the amines in these pesticides as precursors to nitrosamines in tobacco.

Alkaloids present in tobacco may serve as precursors for another class of nitrosamines -- the tobacco-specific nitrosamines. Tobaccos used for commercial products in the United States contain between 0.5% and 2.7% alkaloids. Nicotine constitutes between 85% and 95% of the total alkaloid content (Hecht et al., 1974; Piade and Hoffmann 1980). Important minor alkaloids are nornicotine, anatabine, anabasine, cotinine, and N-formylnornicotine. Several of these alkaloids are secondary and tertiary amines and can be nitrosated. Tobacco-specific nitrosamines identified in tobacco and tobacco smoke include nitroso-nornicotine (NNN), 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and nitrosoanatabine (NAT). In model experiments, nitrosation of nicotine also yielded 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl) butanol (NNA) (Hoffman et al., in press). These nitrosamines are found in tobacco at levels ranging from 1.3 mg/kg to as high as 88 mg/kg (Table 7-4). This wide variation was found to stem from differences in the levels of nitrate and alkaloids in fresh tobacco leaves and the methods used to cure and ferment the tobacco (Hoffmann et al. 1979).

As constituents of chewing tobacco and snuff, carcinogenic tobacco-specific nitrosamines come into direct and prolonged contact with tissues of the oral cavity when the user places and retains the tobacco product between the gum and cheek. Snuff users have a significantly increased risk for oral cancer compared to individuals who do not use tobacco (Pindborg, 1980; Winn et al., 1981a,b). In other studies, a correlation between the increased use of snuff and an increased risk for cancer of the oral cavity has been reported (Christen et al., 1979; Modeer et al., 1980).

Volatile nitrosamines are also formed during smoking, and Hoffmann et al. (in press) have measured a maximum of 70 ng of volatile nitrosamines per cigarette in the mainstream smoke (smoke that passes through the cigarette) in unfiltered U.S. cigarettes (Table 7-5).

TABLE 7-5

Nitrosamines in Mainstream Tobacco Smoke^a

Tobacco Product	Volatile Nitrosamines, ng/Cigarette or Cigar			Pesticide- Derived Nitrosamines, ng/Cigarette or Cigar		Tobacco-Specific Nitrosamines, ng/Cigarette	
	NDMA	NEMA ^b	NDEA	NPYR	NDELA	NAT	NNN
S. cigarettes:							
Cellulose-acetate filter tip	6-7	0.5	1	5-9	24	370	310
Nonfilter	13-27	1-2	1-8	11-33	36	330	240
Charcoal-cellulose-acetate filter tip	14	0.6	8	8	-	-	-
all cigar	-	-	-	-	68	1,700	5,500
large cigar	-	-	-	-	10	1,900	3,200
noncigarettes:							
Cellulose-acetate filter tip	4	0.5	0.1	11	0	190	1,000
Nonfilter	29	2.7	0.6	25	0	640	3,200

^a adapted from Hoffmann et al., in press.
^bEMA = N-nitrosoethylmethylaniline.

filters (Brunnemann et al., 1977; Hoffmann et al., 1980). Another important consideration in determining the exposures of humans to volatile nitrosamines from tobacco smoke is that the concentration of volatile nitrosamines, especially NDMA, in sidestream smoke (smoke from the burning tip of the cigarette) can be as much as 50 times higher than in mainstream smoke (Brunnemann et al., 1980; Hoffmann et al., 1980; Rühl et al., 1980).

In addition to the volatile nitrosamines formed during smoking, each cigarette smoked produces tobacco-specific nitrosamines (Table 7-5). Similar quantities are found in both mainstream and sidestream smoke. The quantities of tobacco-specific nitrosamines in the smoke are dependent on the concentrations of nitrate, nitrite, tobacco alkaloids, and tobacco-specific nitrosamines present in the tobacco itself.

Food

Extensive compilations of the volatile nitrosamine content of foodstuffs in various diets have been published by Gray (in press), Havery et al. (1978), the International Agency for Research on Cancer (1978b), Kawabata et al. (1979), Preussmann et al. (1979), Scanlan (1975), and Schmähl (1980).

Meats. Over the past 9 years, the meat industry and various government and research laboratories have developed techniques to reduce volatile nitrosamines in cooked bacon. Although nitrosamines have not been eliminated, their concentrations have been reduced considerably (Sen et al., 1977b). Data on NPYR, accumulated by the Food and Drug Administration (FDA) from 1971 to 1977, show this reduction clearly (Havery et al., 1978; Table 7-6). The trend toward lower NPYR levels in cooked bacon is partially explained by the use of reduced levels of nitrite and increased levels of the nitrosation inhibitor, ascorbate, in the bacon-curing mixture (Havery et al., 1978). The amount of NPYR formed in cooked bacon is also influenced by the method of cooking, frying temperature, and cooking time (Gray, in press).

In England, Gough et al. (1978) analyzed a variety of foodstuffs typical of the diet in that country. All 50 samples of fried bacon examined contained concentrations of NPYR ranging from 1 to 20 $\mu\text{g/kg}$, and occasionally up to 200 $\mu\text{g/kg}$. In addition, all samples contained nitrosopiperidine (NPIP) (in concentrations up to 0.25 $\mu\text{g/kg}$) and NDMA (in concentrations as high as 5 $\mu\text{g/kg}$). These data are summarized in Figure 7-2.

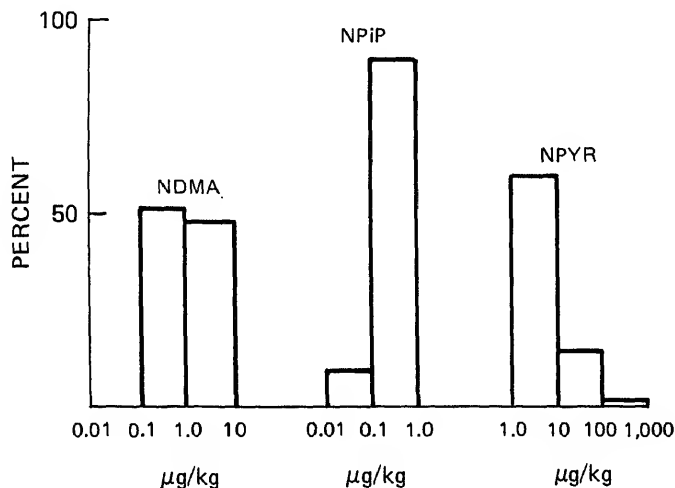
Concentrations of NPYR in Commercial U.S. Cooked Bacon^a

NOTE: Some concentrations have been rounded off to two significant

Year	Concentration ($\mu\text{g/kg}$), by Brand ^b							
	A	B	C	D	E	F	G	H
1971	-	-	-	-	100	77	73	-
1972	110	-	20,13,24	100	-	-	95	-
1973	34	58,36	-	25	39	29	86,79	-
1974	17	-	14,8	7	10	29	96	45,1
1975	-	18	5	12	19	17	65	13,1
1976	18	25	-	3	23	7	33	6
1977	29	14	5	5	12	-	75	10

^aData adapted from Havery et al., 1978.

^bNine brands of bacon purchased at various intervals from 1971 to 1977 in a Washington, D.C. retail market.



samples contained extremely low levels of nitrosamines -- usually less than 1 $\mu\text{g/kg}$. These low levels could be attributed in part to the discontinuation of the use of nitrite-spice premixes in the mid-1970's. Some of those premixes contained NPYR, NPiP, and NDMA (Sen et al., 1974). In England, Gough et al. (1978) found that cured meats other than bacon also contained NPYR, NPiP, and NDMA; however, NPYR did not exceed 1 $\mu\text{g/kg}$. Some cured meats simultaneously contained several nitrosamines in concentrations ranging from 0.1 to 1 $\mu\text{g/kg}$.

Dairy Products. Cheeses of the Gouda and Edam types as produced in certain European countries could contain nitrosamines because of the addition of nitrate to prevent the growth of clostridia (Gray et al., 1979). Gough et al. (1977a) examined 21 different varieties of cheese commonly available in England, including cheeses to which nitrate had been added during manufacture. NDMA was not found more frequently in these samples than in cheese made without added nitrate. This corresponds to the finding that the nitrate content is not higher in cheeses to which nitrate has been added (see Chapter 5). Levels of NDMA were similar for all cheeses (1 to 5 $\mu\text{g/kg}$), except for one sample of Stilton, which contained 13 $\mu\text{g/kg}$. A similar range of concentrations was measured by Sen et al. (1978) in 31 samples of cheese imported into Canada, many of which were known to have been prepared with the addition of nitrate. Havery et al. (1976) failed to detect any of 14 nitrosamines in 17 samples of cheese, 10 of which had been processed with nitrate as an additive.

Fish. Because of its relatively high amine content, fish has been regarded as a likely source of nitrosamines. However, fish products in the United States rarely contain nitrosamines in excess of 1 $\mu\text{g/kg}$.

In a study conducted in England (Gough et al., 1978; Webb and Gough, 1980), approximately 80% of the uncooked and fried fish sampled were found to contain NDMA (Figure 7-3), but among 70 samples tested, the only other nitrosamine detected was NPYR, which was present at a concentration of 0.01 $\mu\text{g/kg}$ in one sample. Five of 24 samples of salted, pickled, smoked, and canned fish also contained NDMA.

The Japanese diet contains a relative large amount of fish, some of which contains high levels of nitrosamines. Table 7-7, taken from Kawabata et al. (1979), shows the NDMA and NPYR levels in various fresh and cooked fish in Japan. These investigators also reported that fish prepared in a gas oven contained higher levels of nitrosamines than fish cooked in an electric oven. Presumably, nitrogen oxides in the combustion products were the source of the nitrosating agents (see Chapter 5). Although

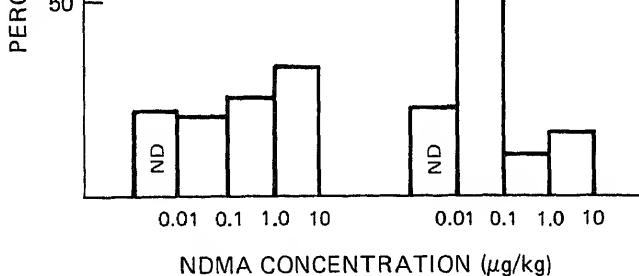


FIGURE 7-3. Measurement of the volatile nitrosamine NDMA in fish (94 samples) and in cheese (76 samples). ND = Not detected. Limit of detection 0.01 µg/kg. From Webb and Gough, 1980.

parallel studies have not been conducted on U.S. foods, it is reasonable to expect that U.S. gas ovens would also lead to elevated nitrosamine levels.

Fruits and Vegetables. Nitrosamine levels lower than 1 µg/kg have occasionally been found in a variety of fruits and vegetables. However, data at these very low levels (<0.2 µg/kg) are unreliable because of the possibility of artifact formation. In one study, 15 baby foods commonly used in the United Kingdom were all free of volatile nitrosamines in concentrations above the detection limit in this assay of 0.01 µg/kg (Webb and Gough, 1980). They also reported that NDMA was found in 5 of 12 samples of canned fruit (< 0.1 µg/kg). Sixteen samples of different varieties of vegetables contained no volatile nitrosamines, and three of 30 soups tested contained NDMA (< 0.09 µg/kg).

Edible Oils. Early reports of the presence of volatile nitrosamines in edible oils have not proven reliable (Hedler and Marquart, 1975; Hedler et al., 1972; White et al., 1974). In a recent paper, Hedler et al. (1979) again reported finding NDMA and NDEA at concen-

anges in Nitrosamine Content of Japanese Salt-Dried Fish and Shellfish after Broiling a

NOTE: Some of the concentrations have been rounded to two significant figures.

Sample	Method of Broiling	Nitrosamine Content, $\mu\text{g/kg}$		Sample	Method of Broiling	Nitrosamine Content, $\mu\text{g/kg}$	
		NDMA	NPYR			NDMA	NPYR
Salt-dried horse mackerel (aji-hiraki)	(1) ^b Raw ^c	1.0	NR ^d	Salt-dried	Raw	0.5	NR
	Gas ^e	6.0	NR	chub mackerel (saba-hiraki)	Al. foil ^f Electric ^g Gas	5.8 7.3 12	NR NR NR
	(2) Raw Gas	2.4 7.8	NR NR	Salt-dried round herring (urume-iwashi)	Raw Al. foil Electric Gas	5.0 2.5 4.5 26	NR NR NR NR
Salt-dried shishyamo	(3) Raw Al. foil Electric Gas	4.9 3.4 5.9 9.4	NR NR NR NR	Dried squid	(1) Raw Gas	15 24	NR 7.2
	(1) Raw Gas	0.5 1.0	NR NR		(2) Raw Gas	58 140	NR 2.4
Salt-dried Pacific saury (sauma-hiraki)	Raw Al. foil Electric Gas	4.0 13 Tr ^h 3.7 1.1 6.8	NR NR NR NR NR NR		(3) Raw Gas	84 310	NR 9.7
					(4) Raw Gas	56 110	NR 13
					(5) Raw Al. foil Electric Gas	18 19 49 69	NR NR 3.7 3.3

^aAdapted from Kawabata et al., 1979.

^bNumbers presumably refer to different analyses of the same species of fish.

^cRaw = uncooked fish.

^dNR = no data reported.

^eGas = broiled in a city gas range.

^fAl. foil = samples covered with aluminum foil broiled in a city gas range.

^gElectric = broiled in an electric range.

^hTr = trace.

tations as high as 23 and 28 $\mu\text{g/kg}$, respectively, in one-third of 61 samples. This finding has not been confirmed and is presumed to be due to artifact formation (Preussmann, 1980). More recently, Fiddler et al. (1981) studied 21 edible oils and reported NDMA at levels of only 0.22 to 1.0 $\mu\text{g/kg}$.

Alcoholic Beverages. Considerable attention has been focused over the past few years on the presence of nitrosamines in beer and other alcoholic beverages. Spiegelhalder et al. (1979) analyzed 158 samples of different types of beer in the Federal Republic of Germany and reported that 70% of them contained NDMA (mean concentration, 2.7 $\mu\text{g/kg}$). Goff and Fine (1979) reported NDMA levels ranging from 0.4 to 7.0 $\mu\text{g/kg}$ in 18 brands of U.S. and imported beers. In addition, six of seven brands of Scotch whiskey, which is also made from malt, contained NDMA at levels between 0.3 and 2.3 $\mu\text{g/kg}$. Scanlan et al. (1980) reported NDMA in 23 of 25 beer samples, at levels ranging from 0 to 14 $\mu\text{g/kg}$ and averaging 5.9 $\mu\text{g/kg}$.

In a study conducted by the German Cancer Research Center, Spiegelhalder et al. (1980a,b) analyzed 215 beer samples in the Federal Republic of Germany, 141 of which contained NDMA. The mean NDMA concentration in beer was 2 to 5 $\mu\text{g/liter}$; one sample of a beer made with smoked malt (Rauchbier) contained 68 $\mu\text{g/liter}$. NDEA was detected in only 2 of the 215 beer samples, at levels of 3.0 and 0.5 $\mu\text{g/liter}$. The levels of NDMA found by these investigators in different types of beer in the Federal Republic of Germany and in other European countries are shown in Tables 7-8 and 7-9. Stephany and Schuller (1980) who also analyzed 57 samples of beer consumed in the Netherlands, found that 72% contained NDMA at levels ranging from 0 to 5.7 $\mu\text{g/liter}$.

Following the initial reports of nitrosamines in beer, efforts were made to determine the source(s) of these contaminants. The only significant source of NDMA was found to be the malt, which contains a variety of amine precursors including hordenine, gramine, and methyltyramine (Mangino et al., in press), that had been exposed to nitrogen oxides during the drying process (Scanlan et al., 1980; Spiegelhalder et al., 1980a,b). Consequently, changes in malting procedures were implemented, resulting in markedly reduced nitrosamine levels in both malts and beer (Havery et al., 1981; Preussmann et al., 1980, in press b). For example, since sulfur dioxide or products of sulfur combustion were found to reduce exposure of the malt to nitrogen oxides, sulfur was added to the open flame used to dry the malt, which led to reduced formation of nitrosamines (Preussmann et al., 1980). In addition, Preussmann et al. (1980) reported that the use of a burner that reduces nitrogen oxide synthesis resulted in the production of malt containing NDMA concentrations as low as 1 to 3 $\mu\text{g/kg}$ -- a 15- to 30-fold reduction.

NDMA in Different Types of Beer
in the Federal Republic of Germany^a

<u>Type of Beer</u>	<u>No. of Samples</u>	<u>% positive (> 0.5 µg/liter)</u>	<u>Mean, µg/liter</u>	<u>Maximum, µg/liter</u>
Top fermented pale ales	22	23	0.2	1
Alcohol-free and diet lager beers	16	69	1.0	4
Pale lager and export lager	42	67	1.2	7
Pilsen lager	54	65	1.2	7
Pale strong lager	25	76	1.9	8
Top fermented dark ales	25	76	2.7	11
Dark lager and dark strong lager	22	68	6.0	47
Rauchbier (from smoked malt)	9	100	18	68
All types	215	66	2.5	68

^aFrom Spiegelhalder et al., 1980b.

TABLE 7-9

NDMA Levels in European Beers^a

<u>Country</u>	<u>No. of Samples</u>	<u>Mean, µg/liter</u>	<u>Maximum, µg/liter</u>
Austria	12	3.0	10
Belgium	16	1.3	12
Denmark	5	0.5	0.5
France	21	1.7	7.0
German Democratic Republic	11	0.6	1.0
Great Britain and Ireland	14	2.7	8.0
Soviet Union	5	1.0	2.5
Sweden	5	0.3	0.8
Switzerland	10	1.6	9.0

^aFrom Spiegelhalder et al., 1980b.

undetectable levels (0.2 or 0.4 $\mu\text{g/liter}$ to 7 $\mu\text{g/liter}$), averaging less than 1 $\mu\text{g/liter}$. NDMA in 80 samples of imported beers ranged from undetectable levels (0.2 or 0.4 $\mu\text{g/liter}$) to 13 $\mu\text{g/liter}$, averaging 1 $\mu\text{g/liter}$ (Havery et al., 1981).

Walker et al. (1979) and Goff and Fine (1979) screened other alcoholic beverages such as brandy, rum, wine, and cider. Goff and Fine were not able to detect volatile nitrosamines in U.S. beverages other than beer and Scotch whiskey. Walker and colleagues did find volatile nitrosamines in 74 of 145 French apple brandies and cognac at concentrations generally ranging from 0.18 to 0.6 $\mu\text{g/kg}$. The highest concentration found by these investigators was 10 $\mu\text{g/kg}$ in an apple brandy. They also found nitrosamines in 7 of 17 rums (average, 0.3 $\mu\text{g/kg}$), in 3 of 4 kirsch samples (average, 3.2 $\mu\text{g/kg}$), and in 2 of 2 mirabelle samples (average, 3.8 $\mu\text{g/kg}$).

Cosmetics

Many cosmetics, soaps, and shampoos are contaminated with NDELA (Fan et al., 1977a). The source of the amine is triethanolamine, which is present in most cosmetic formulations (see Chapter 6). Bactericides such as 2-bromo-2-nitro-1,3-propanediol (bronopol) are generally considered to be the nitrosating agents (Fan et al., 1978a; Ong and Rutherford, 1980). NDELA levels in cosmetics have been found to range from less than 1 $\mu\text{g/kg}$ to 48,000 $\mu\text{g/kg}$ (Fan et al., 1977a).

The presence of nitrosamines in products containing lauramine oxide was analyzed by combined GC-TEA and HPLC-TEA (Hecht, 1981). Of seven products analyzed, six gave positive responses on both HPLC-TEA and GC-TEA for nitrosododecylmethylamine (NDOMA). The peak corresponding to NDOMA decreased upon photolysis, a reaction characteristic of nitrosamines. One sample was analyzed on a large scale, and the mass spectrum obtained was characteristic of NDOMA. The concentrations of NDOMA in these products ranged from 0.02 to 0.60 $\mu\text{g/kg}$. Apparently, some NDOMA is also present in the lauramine oxide ingredient itself. These results show that NDOMA, a bladder carcinogen, is present in cosmetics formulated with lauramine oxide.

Analyses for nitrosobenzylmethylamine (NBMA) and nitrosomethylstearylamine (NMSA) in products containing stearylalkonium chloride are currently in progress in Hecht's laboratories. Of eight samples screened by combined HPLC-TEA, three gave a positive response for NMSA and, tentatively, NBMA. Further work is needed to confirm the

indicating the presence of other nitroso compounds, nitro compounds, or nitrite esters (Hecht, 1981).

Pharmaceuticals

Many drugs contain chemically bound nitrogen and have sites for possible nitrosation. For example, cimetidine, i.e., N-cyano-N'-methyl-N''-[2-[(5-methyl-1H-imidazol-4-yl)methyl]thio]ethyl]guanidine which is a drug used for treatment of peptic ulcers, has received considerable attention recently, and its possible nitrosation products are being investigated (Henderson and Basilio, 1980; Jensen and Magee, 1981). In addition, vasodilators derived from nitrate or nitrite esters can behave as potential nitrosating agents. Nitrosation may occur exogenously (within the drug itself) or it may occur endogenously in humans after ingestion of the drug (see discussion in Chapter 8).

Eisenbrand et al. (1979) analyzed 68 commercial formulations of the drug aminopyrine for the presence of preformed nitrosamines. Although this drug was available in the Federal Republic of Germany, none of the formulations had been licensed for sale in the United States. All formulations contained NDMA in amounts varying from 1 to 370 $\mu\text{g/kg}$. These investigators also demonstrated that aminopyrine, a tertiary amine, reacts extremely rapidly with trace levels of nitrogen oxides in the air to form the NDMA.

Krull et al. (1979b) reported that nitrosamine impurities were absent from 68 of 73 pharmaceutical products, consisting of both prescription and over-the-counter drugs available in the United States. The methods used by these investigators were designed to detect both volatile and nonvolatile N-nitroso compounds at levels as low as 1 $\mu\text{g/kg}$.

Pesticides

Fan et al. (1976) reported that nitroaniline herbicides, and formulations prepared as the amine salt, contained substantial nitrosamine impurities. For example, a formulation of the herbicide trifluralin, i.e., α,α,α -trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine, was shown to contain 154 mg of nitrosodipropylamine (NDPA) per liter (Ross et al., 1977). Two experimental field studies have demonstrated that the nitrosamine impurity in trifluralin is not detectable as an air pollutant on farms, even during application, presumably because the pesticide is generally applied by incorporation into the soil (Ross et al., 1977; West and Day, 1979). Further-

nitration with nitric and sulfuric acids was followed by the addition of dipropylamine (Fine et al., 1977b). If the residual nitrosating agent were still present during the addition of dipropylamine, then NDPA would be formed as an impurity. Within a few months after the discovery of the NDPA impurity, the manufacturer was able to reduce it 10-fold to 18 mg/liter (Fine, 1980b). The impurity has been further reduced to less than 1 mg/liter (Zweig and Garner, in press) by eliminating the nitrogen oxides formed during a nitration step before the synthesis step in which dipropylamine is introduced.

Similar success has been achieved in the reduction of nitrosamine contaminants in amine salt pesticides. Some dimethylamine salt formulations of 2,3,6-dichlorobenzoic acid had contained NDMA levels as high as 187, 195, and 640 mg/liter. The source of the nitrosating agent was traced to metal cans treated with sodium nitrite. A switch to plastic-lined cans reduced the NDMA concentration to less than 2 mg/liter.

Work by the EPA has extended the study of nitrosamine impurities in pesticides to cover more than 300 formulations (Bontoyan et al., 1979; Cohen et al., 1978; Zweig and Garner, in press). Analytical procedures that have been used to screen pesticide formulations for nitrosamines have been reviewed by Wolf et al. (1980). From these analyses, it was determined that only the following chemical classes of pesticides contained identifiable N-nitroso contaminants:

- substituted dinitroaniline derivatives
- dimethylamine salts of phenoxyalkanoic acid herbicides
- di- and triethanolamine salts of several pesticides
- some quaternary ammonium compounds
- dimethyl thiocarbamoyl disulfide (thiram)
- some morpholine derivatives

The EPA analyses of these pesticides showed that concentrations of N-nitroso compounds ranged from 1.2 to 430 µg/liter. The chemical identity of the contaminants was usually predictable on the basis of the chemical structure and route of synthesis of the pesticide compound. For example, pendimethalin [N(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine] yields nitrosopendimethalin, 2,4-D dimethylamine salt yields NDMA, and dinoseb triethanolamine salt yields NDELA. Many such nitrogen-containing pesticides were subsequently analyzed and found to contain less than 1 µg/liter of the corresponding N-nitroso compound contaminant (Zweig et al., 1980). Results of these analyses are tabulated in Table 7-10. The EPA has proposed a policy for

Pesticides Contaminated with N-Nitroso Compounds in Concentrations
Lower than 1 µg/liter^a

<u>Chemical Class</u>	<u>Pesticide</u>
Amides	Alachlor Clonitralid Diphenamid
Carbamates and thiocarbamates	Carbaryl Ethiofencarb Methiocarb Propoxur Sulfallate Triallate
Triazines	Ametryn Anilazine Atrazine Cyanazine Metribuzin Propazine Simazine
Organophosphates	Acephate Azinphos-methyl Azodrin Dialifor Dicrotophos Fenamiphos Isofenphos Methamidophos Methyl parathion
Substituted ureas	Diuron Linuron
Miscellaneous classes	Chlorothalonil Fenaminosulf Maleic hydrazide Oryzalin Oxythioquinox Paraquat

to provide an example of how human exposure to N-nitroso compounds may be reduced.

The analysis of the N-nitroso compound content of the following classes of compounds would be required:

- dinitroanilines
- secondary and tertiary alkylamines, arylamines, and alkanolamines
- quaternary ammonium compounds, if a nitrosating agent such as nitrite has been added

Furthermore, the EPA would set the level of detection of nitrosamines at 1 mg/liter. This level is not based on the sensitivity of present instrumentation (e.g., the TEA can detect levels of N-nitroso compounds as low as 0.01 to 0.02 mg/liter) but, rather, on the practicality of directly and routinely analyzing formulations without extraction or clean-up. Moreover, because commercial formulations are diluted, 1 mg/liter concentrations of N-nitroso compounds in the technical grade material would be correspondingly lower in the final product.

According to the proposed EPA policy for products containing any of the above classes of compounds for use in the United States, registrants must submit to the EPA analytical data on the possible nitrosamine content of their products. Samples for analysis must be obtained from fresh production batches. If a nitrosating agent is present in or added to the final product, the same sample must be reanalyzed following storage at room temperature for 3 and 6 months. (This requirement may be slightly modified in the final regulations to reduce the number of samples.) If the analyses demonstrate that nitrosamine contamination does not exceed 1 mg/liter, the product is cleared for registration.

When nitrosamine concentrations exceed 1 mg/liter, the EPA proposals require that the registrant provide data on the carcinogenic potential of the contaminant and estimate potential exposure for persons coming into contact with the pesticide during normal application procedures and farm practices. On the basis of the exposure analysis and potential carcinogenic risk, the EPA staff will perform a risk estimation according to statistical methods such as linear extrapolation (one-hit model) or multistage analysis (Fishbein, 1980).

The EPA proposals suggest that the acceptable level of risk for N-nitroso compounds is approximately 10^{-6} . This level will be used by the agency to distinguish between high-risk (greater than

the EPA proceeds on a regulatory action, registrants whose products fall into the high-risk category, i.e., exceed the 10^{-6} risk level, will be given the opportunity to lower potential exposure, thereby reducing the risk to users of their pesticides.

Suggested methods for reducing the nitrosamine content of the product include modification of the manufacturing process. This can be accomplished by using nitrosamine-free starting materials or intermediates or by eliminating or reducing all potential nitrosating agents formed or added during the manufacturing process (e.g., nitrous acid resulting from nitration). Nitrosating agents may also be eliminated by improving packaging technology. For example, the use of sodium nitrite as a corrosion inhibitor for metal containers could be discontinued or the metal containers could be lined with plastic to prevent exposure of pesticide formulations to metal treated with sodium nitrite. Exposure can also be reduced by modifying application techniques or by eliminating high-exposure uses (U.S. Environmental Protection Agency, 1980).

Water

Water is deionized with ion exchange resins for use in high-pressure steam production, chemical and food processing, mining, agriculture, laboratories, automobile batteries, steam irons, and, to a limited degree, in drinking water in areas with water of high salinity. Nitrosamines have been observed in deionized water by Fiddler et al. (1977), Cohen and Bachman (1978), and Gough et al. (1977b). Fiddler et al. (1977) reported that 13 of 19 samples of water exposed to regenerated deionizing resins contained NDMA in concentrations ranging from 0.03 to 0.34 $\mu\text{g}/\text{kg}$. NDMA concentrations of 0.25 $\mu\text{g}/\text{kg}$ and lower were also detected in deionized water by Cohen and Bachman (1978). The mechanism of nitrosamine formation in deionized water is not yet clearly understood (Angeles et al., 1978; Gough et al., 1977b; Kimoto et al., 1980). Volatile nitrosamines such as NDMA have also been identified in industrial wastewater in concentrations ranging from 0.2 to 5 $\mu\text{g}/\text{liter}$ (Cohen and Bachman, 1978; Fine et al., 1977b). In well water with a high nitrate content, NDEA and NDMA have been found at levels lower than 0.01 $\mu\text{g}/\text{liter}$ (Fine and Rounbehler, 1976).

Volatile nitrosamines have been shown to be absent in drinking water in Baltimore, Md. (Fine et al., 1977c); Belle, W. Va.; Boston and Waltham, Mass. (Fine et al., 1977a); New Orleans, La. (Fine and Rounbehler, 1976); Cincinnati, Ohio; Philadelphia, Penna.; Washington, D.C. (Fan et al., 1980); Kansas City, Ks.; Lexington, Mo.; and

Air

Amines and oxides of nitrogen can react to form nitrosamines (see Chapter 4). This reaction is partially responsible for the nitrosamine contamination of workroom air in tanneries and rubber factories (see earlier discussion on occupational settings).

NMOR and NDMA have been found in the interior air of new 1979 model automobiles by Rounbehler et al. (1980). In the 38 automobiles tested, the concentrations ranged from 0.07 to 0.83 $\mu\text{g}/\text{m}^3$ (average, 0.3 $\mu\text{g}/\text{m}^3$) for NDMA, from 0.07 to 2.5 $\mu\text{g}/\text{m}^3$ (average, 0.67 $\mu\text{g}/\text{m}^3$) for NMOR, from 0.04 to 0.39 $\mu\text{g}/\text{m}^3$ (average, 0.11 $\mu\text{g}/\text{m}^3$) for NDEA, and trace levels (less than 0.01 $\mu\text{g}/\text{m}^3$) for nitrosodi-N-butylamine (NDBA). The source of the nitrosamines in at least one car was shown to be the spare tire. Other sources contributing to the presence of NMOR and NDMA may include rubberized mats, rubber grommets, and rubber sealants.

NDMA pollution of indoor air from the burning of tobacco was investigated by Brunnemann and Hoffmann (1978). The appreciable amounts of NDMA detected in these environments were attributed largely to sidestream smoke.

A large portion of the NPYR produced during the frying of bacon escapes with the fumes (Sen et al., 1976; also see review by Gray, in press). Fine (1980b) has calculated that a person inhaling the nitrosamine-laden air for 30 minutes in a typical domestic kitchen would be exposed to no more than 0.12 μg of NPYR.

In studies conducted in New York City, Boston, and northern New Jersey, Fine et al. (1977a) found little evidence to suggest that NDMA or other N-nitroso compounds were being formed in the atmosphere, even near amine factories. NDMA was found sporadically at only three of the 40 sites studied. These results suggest that airborne NDMA and other N-nitroso compounds do not present a widespread air pollution problem, but, rather, that they are localized pollutants associated with specific environments. One reason for this may be due to the rapid breakdown of N-nitroso compounds following exposure to light (Hanst et al., 1977).

EXPOSURE OF HUMANS TO N-NITROSO COMPOUNDS

Individual Exposures, by Source

tanning industry where up to 440 μg of NDMA may be inhaled daily (Fine, 1980a).

The exposure of humans to NMOR in rubber factories was studied by McGlothlin et al. (1981). In this study, an area air sample contained 250 $\mu\text{g}/\text{m}^3$ of NMOR, and the highest personal (breathing zone) concentration was measured at 25 $\mu\text{g}/\text{m}^3$ (time-weighted average) for a worker in the feed mill and calender operation, a so-called "hot-process area" where rubber is heated by friction and compression. Daily exposure at this concentration of NMOR would equal 250 μg . In another study of exposures in the rubber industry, daily exposure to NDPhA ranged from 5 μg to 430 μg (Fajen et al., 1979). Based on concentrations of NDMA and NMOR in rubber factories located in the Federal Republic of Germany, Preussmann et al. (1980) calculated that the average daily exposure to these nitrosamines was approximately 50 μg . More recently, Spiegelhalder and Preussmann (in press) reported that personal breathing zone samples in injection molding and curing areas of an industrial rubber products factory contained NMOR at an average concentration of 380 $\mu\text{g}/\text{m}^3$ and NDMA at an average concentration of 90 $\mu\text{g}/\text{m}^3$ (average over an 8-hour period). These concentrations would result in daily intakes of 3.8 μg NMOR and 0.9 μg NDMA. The only other data on occupational exposures to nitrosamines were reported by Fine et al. (1976b,c) in their studies of NDMA levels in a rocket fuel factory. Daily exposures of factory workers to NDMA from this source were calculated to be a maximum of 260 μg (average, 10 to 50 μg).

TABLE 7-11

Occupational Exposure to Airborne Nitrosamines^a

Industry	Maximum Daily Exposure, $\mu\text{g}/\text{Person}/\text{Day}^b$		
	NDMA	NMOR	NDPhA
Leather tanning	440	20	
Rubber (tire curing)		250	
Rubber chemical production			430
Rocket fuel production	260		

^aAdapted from Fine, 1980a.

^bIt is assumed that an average worker weighs 70 kg (1.65 m^2 body surface area) and breathes 30 liters/min

tobacco smoke.

Based on the levels of nitrosamines measured in snuff and chewing tobacco (see Table 7-4), exposure of humans to nitrosamines from these sources probably exceeds exposure from cigar and cigarette smoke. In addition to exposure to preformed tobacco-specific nitrosamines, in vitro and in vivo experiments have indicated that humans can also be exposed to tobacco-specific nitrosamines that are formed during snuff dipping (Hecht et al., 1974). A comparison of the saliva of snuff dippers and tobacco chewers shows that tobacco-specific nitrosamines are present in saliva in a wide range of concentrations. The variation is ascribed to differences in the product, in the manner of chewing, and the composition of each person's saliva (Hoffmann and Adams, in press). Data on concentrations of tobacco-specific nitrosamines in the saliva of snuff dippers measured by Hoffmann et al. (in press) are presented in Table 7-12. Combined nitrosamine levels of 600 ng/g in the saliva seem fairly common. Thus, 100 ml of saliva from a snuff dipper would contain 60 µg of the tobacco-specific nitrosamines.

TABLE 7-12

Tobacco-Specific Nitrosamines in Saliva of Snuff-Dipping Women^a

NOTE: Some of the concentrations have been rounded off to two significant figures.

Age	Tobacco-Specific Nitrosamines, ng/g		
	NAT	NNN	NNK
37	17-510	30-130	13
40	29	22	2
41	210-470	110-140	21-26
43	14	27	20
44	320-370	320-420	62-96
45	7	5	8
52	13-46	26-57	11-23
53	150	120	200

^aData adapted from Hoffmann et al., in press.

Tobacco smoke has been shown to contain eight nitrosamines: NDELA, four volatile nitrosamines (NDMA, NEMA, NDEA, and NPYR), and three tobacco-specific nitrosamines (NAT, NNN, and NNK). In estimating the exposure of humans to these compounds, several facts must be considered. First, the amount of tobacco-specific nitrosamines present in cigarettes far exceeds the amount of NDELA or of the volatile nitrosamines. Second, the cellulose acetate filter tips seem to trap a large portion of the volatile nitrosamines. Third, the amount of tobacco-specific nitrosamines is almost 10 times greater in the smoke of cigars than of cigarettes. Exposure of humans can be estimated by adding the concentrations given in Table 7-5 for all nitrosamines present in tobacco smoke. The nitrosamine intake is 0.87 μg for a U.S. cellulose-acetate filter tip cigarette, 0.76 μg for a U.S. nonfilter cigarette, 1.4 μg for a French cellulose-acetate filter tip cigarette, 4.3 μg for a French nonfilter cigarette, and as high as 11 μg for a small cigar. If these data are assumed to be typical of an average cigarette, then a pack of 20 U.S. filter cigarettes represents an intake of approximately 17 μg .

Food. Dietary exposures to preformed nitrosamines have been assessed for typical residents of the United Kingdom (Gough et al., 1978; Webb and Gough, 1980), the Netherlands (Stephany and Schüller, 1980), and the Federal Republic of Germany (Spiegelhalder et al., 1980a,b). A similar food-by-food assessment of dietary intake of nitrosamines has not yet been undertaken in the United States.

In the United Kingdom, the Laboratory of the Government Chemist (Gough et al., 1978) conducted extensive analyses of foodstuffs typical of the diet of the U.K. population. Foods purchased in normal retail outlets were analyzed while within their shelf life or, when appropriate, after cooking. Tested foods included bacon, canned meats, fresh meat and meat products, fish and fish products, cheese, yogurt, desserts, canned fruits and jams, frozen and fresh vegetables, soups, beverages (but not beer), and baby foods. Complete meals were prepared under normal domestic conditions, and then the nitrosamine content of the prepared foods was measured. The investigators found that cured meats were the major source of volatile nitrosamines and that fish and cheese were the second and third largest sources. All other foods examined contained an average nitrosamine concentration less than 0.06 $\mu\text{g}/\text{kg}$.

The intake of the nitrosamines was estimated by determining the average intake and the nitrosamine content of the various food items consumed daily (Table 7-13). The investigators concluded that the likely daily intake of volatile nitrosamines from the normal diet (excluding beer) is 0.53 μg and that 0.43 μg of that

NOTE: Some of the numbers in this table have been rounded off to two significant figures.

<u>Food</u>	<u>Food Consumption, g/Person/Day</u>	<u>Total Nitrosamine Intake, µg/Person/Day</u>
Cured meats	49	0.43
Fish	20	<0.01
Cheese	14	<0.01
All other foods	1,400	0.08
TOTAL	1,500	0.53

^aFrom Gough et al., 1978.

The National Institute of Public Health in Bilthoven, the Netherlands, studied the nitrosamine content of 206 foods and beverages ingested by volunteers over a 24-hour sampling period (Stephany and Schuller, 1980). Based on the concentrations of nitrosamines found in various foods (Table 7-14), the average daily intake of NDMA was calculated to be 0.38 µg. In diets containing beer, beer was shown to be the major dietary source of NDMA, contributing 90% of the total NDMA intake of 1.1 µg. Because of the major contribution of beer and because males generally drink more beer than females, NDMA intake by the average male was approximately 4 times greater than that of the average female. However, since that study was undertaken, maltsters have modified their processes to reduce the nitrosamine contamination of malt.

The German Cancer Research Center conducted a survey of the nitrosamine levels in 2,826 commercially produced food products (Spiegelhalder et al., 1980a,b). Among the products tested were cured meats, cheeses, and numerous beer samples, along with more than 2,000 other types of food not normally associated with volatile nitrosamines. Data on these foods were then used to estimate typical concentrations to which humans are exposed. The calculations were based on the average per capita consumption representative of males in the Federal Republic of Germany. The average daily intakes of volatile nitrosamines from food and beverages in 1971-75 were 1.1 µg.

<u>Food</u>	<u>Samples</u>	<u>Mean</u>	<u>Range</u>	<u>Samples^b</u>
Beer	57	1.2	0 ^c -5.7	72
Whiskey	7	0.3	0-0.9	86
Cured meat	38	0.5	0-3.6	71
Veal	22	0.1	0-0.4	29
Seafood	53	0.4	0-2.1	55
Cheese	84	0.1	0-1.1	45

^aFrom Stephany and Schuller, 1980.

^bSamples with content equal to or higher than the limit of detection (0.1 µg/kg).

^c0 = Content below limit of determination.

TABLE 7-15

Average Daily Intake of Nitrosamines by Males in the Federal Republic of Germany. Calculated from the Average per Capita Consumption^a

<u>Food</u>	<u>Product Consumption, g</u>	<u>Nitrosamine</u>	<u>Nitrosamine Intake, µg Per Capita</u>
Beer ^b	560	NDMA	0.7
Meat and meat products	210	NDMA	0.1
		NPYR	0.1
		NPiP	0.01
Cheese	30	NDMA	0.01
Others	1,500	NDMA	0.2
		NPYR	0.03
TOTAL	2,300	NDMA	1.1
		NPYR	0.13

^aData adapted from Spiegelhalder et al., 1980b.

^bNDMA intake is corrected to account for the proportion of sales for different types of beer.

Percentage Contributed by Different Types of Food to Total NDMA
Intake (1.1 $\mu\text{g}/\text{person}/\text{day}$) by Males in the
Federal Republic of Germany^a

<u>Food</u>	<u>Total Percentage of NDMA Intake</u>	<u>Percentage of Total Food Consumption (by weight)</u>
Beer	64	24
Meat and meat products	10	9
Cheese	1	1
Others	25	66

^aFrom Spiegelhalder et al., 1980b.

NDMA, and all other products together were responsible for approximately 0.2 μg of NDMA and 0.03 μg of NPYR. The total daily intake by males was 1.1 μg of NDMA and 0.13 μg of NPYR.

At the time of this study, the largest contribution was clearly made by beer, which accounted for approximately 64% of the total NDMA intake (Table 7-16). But as stated earlier, maltsters have modified their manufacturing processes so that current NDMA levels in European beers average 1 $\mu\text{g}/\text{liter}$ (Havery et al., 1981).

Estimated Exposure of the U.S. Population to Nitrosamines

The committee has developed estimates of possible exposures to nitrosamines from the various sources in the environment discussed earlier in this chapter. Because of the variation in the concentrations of nitrosamines detected in each of the sources listed in Table 7-17, no attempt has been made to present an overall average concentration of these chemicals in each source. Instead, the committee has selected values that have been published by groups who have made comprehensive surveys of each environmental medium.

tions concerning average consumption and usage of the various products (Table 7-17) in order to develop estimates of exposure of humans. As a result, the exposure levels given in Table 7-17 should not be interpreted as absolute numbers but, rather, should be used to understand the relative importance of the various environmental sources of nitrosamines. For example, the numbers in Table 7-17 indicate that tobacco smoke contributes far more to the exposure of humans to nitrosamines than any other source included in this table (occupational exposure would be higher, but it is not included).

Nonsmokers would be exposed to nitrosamines (in descending order) in beer (0.97 or 0.34 μg), automobile interiors (0.50 or 0.20 μg), in cosmetics (0.41 μg), in bacon (0.17 μg), and in Scotch whiskey (0.03 μg). However, these comparisons of relative exposures are made among different nitrosamines (that vary widely in their carcinogenic potential) and different exposure routes (e.g., inhalation, ingestion, and dermal contact). Thus, a further word of caution concerning the overinterpretation of these data seems necessary since it is presently unknown whether carcinogenic potency in animals (see Chapter 9) is similar to that in humans and whether one route of exposure is more important in the production of adverse health effects.

Two other sources of exposure were not included in this discussion -- occupational environments and endogenous formation of nitrosamines. Certain occupational environments have, however, been discussed in detail earlier and may result in extremely high exposures (e.g., 440 $\mu\text{g}/\text{day}$ in a U.S. tannery). The possible contribution to the exposure of humans resulting from the endogenous synthesis of nitrosamines is discussed in Chapter 8.

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Humans may be exposed to N-nitroso compounds as preformed nitrosamines in the environment via inhalation, ingestion, and dermal contact or following their formation in the body from various precursors. Because analytical methods for volatile nitrosamines are sufficiently sensitive, the levels of these compounds in many environmental sources have been determined. However, adequate methods for unstable and/or nonvolatile N-nitroso compounds have only recently been developed.

Nitrosamines have been detected in occupational settings, such as rubber factories and leather-tanning operations, and in

Source of Exposure	Nitrosamine	Primary Exposure Route	Concentration	Daily Intake µg/person
Cigarette smoking ^a	NDEA	Inhalation	1.0 ng/cig)	17
	NEMA	Inhalation	0.5 ng/cig)	
	NDMA	Inhalation	6.5 ng/cig)	
	NPYR	Inhalation	7 ng/cig)	
	NDELA	Inhalation	24 ng/cig)	
	NNN	Inhalation	310 ng/cig)	
	NAT	Inhalation	370 ng/cig)	
	NNK	Inhalation	150 ng/cig)	
Automobile Interiors	NDMA)	Inhalation	1.0 µg/m ^{3b}	0.50 ^b
	NMOR)		0.34 µg/m ^{3c}	0.20 ^c
Beer	NDMA	Ingestion	2.8 µg/liter	0.97 ^d
			1.0 µg/liter	0.34 ^e
Cosmetics ^f	NDELA	Dermal	11 mg/kg	0.41
Cured meat; cooked bacon ^g	NPYR	Ingestion	5 µg/kg	0.17
Scotch whiskey ^h	NDMA	Ingestion	0.97 µg/liter	0.03

^aAverage concentrations were taken from Hoffmann et al. (in press). See Table 7-5.

^bEstimate represents exposures from new automobile interiors. Average nitrosamine concentration was taken from Rounbehler et al., 1980. The committee assumed an average daily exposure of approximately 1 hour/day.

^cThe committee has assumed that the average daily exposure is approximately 1 hour/day and that it occurs in both new and older automobiles. Since the nitrosamine concentration is likely to be lower in older automobiles, the committee has reduced the average concentration to one-third of that in new automobiles.

- liters of domestically produced beer were sold in the United States (United States Brewer's Association, 1980). The committee has assumed 75% of the population consumed beer and the yearly per capita consumption was approximately 126 liters.
- ^eThe average NDMA content of U.S. beers is currently 1.0 $\mu\text{g}/\text{liter}$ (Havery et al., 1981). The committee has used the above per capita consumption figure of 126 liter per year (see footnote d).
- ^fThe average NDELA level in cosmetics (11.3 mg/kg) was calculated by averaging the seven data points in Fan et al. (1977a). If a woman uses 2 g of cosmetics per day, then 22.6 μg NDELA would be applied to the skin and approximately 1.8% of this would penetrate (Edwards et al., 1979).
- ^gIn 1979, approximately 1.5 billion pounds (~680 million kg) of bacon was produced in the United States (American Meat Institute, 1980). Assuming 25% of the U.S. population (55 million people) consumes bacon, daily per capita consumption would be 0.034 kg. The U.S. Department of Agriculture requires that bacon contain less than 10 $\mu\text{g}/\text{kg}$ nitrosamines. The committee has assumed that bacon contains one-half this amount (5 $\mu\text{g}/\text{kg}$).
- ^hThe average NDMA content of Scotch whiskey was calculated by averaging the data from seven samples analyzed by Goff and Fine (1979). It is assumed that the average drinker consumes 30 ml per day.

cosmetics, pharmaceuticals, pesticides, automobile interiors, water, and air. These data, coupled with assumptions concerning lifestyle and population behavior (e.g., number of cans of beer consumed per day, amount of cosmetic used, occupation, and hours in a car provide a basis for estimating exogenous exposure of humans to nitrosamines.

Because large quantities of nitrosamines are formed in certain occupational settings and are present in tobacco and tobacco smoke, humans may be exposed to high concentrations of nitrosamines from these sources. For example, a maximum exposure to NDMA was estimated to be 440 $\mu\text{g}/\text{day}$ in a leather-tanning facility. In one study of the U.S. rubber industry, a maximum exposure to NMOR of 250 μg was reported and maximum exposure of humans to NDMA from a rocket fuel factory was calculated to be 260 $\mu\text{g}/\text{day}$. However, the figures for the tanning facility and the rocket fuel factory are maximum possible exposures estimated from area air samples. Exposure of workers could be

approximately 10% of these values if their typical activities over an 8-hour day are considered. Breathing zone samples would provide a more accurate indication of actual exposures.

Summing the levels of all nitrosamines present in tobacco smoke, total concentration is 0.87 μg per U.S. cellulose-acetate filter tip cigarette, 0.76 μg per U.S. nonfilter cigarette, 1.4 μg per French cellulose-acetate filter tip cigarette, 4.3 μg per French nonfilter cigarette, and as high as 11 μg for a small cigar. Smoking a pack of 20 U.S. cellulose acetate filter cigarettes would result in an intake of approximately 17 μg .

Assays of foodstuffs in the Netherlands and the Federal Republic of Germany have indicated that the largest single dietary source of nitrosamines is beer, which, in the German study, contributed 64% of the volatile nitrosamines in the diet. Since this study was completed, however, the concentrations of nitrosamines in beer have decreased considerably. Cured meat and meat products contribute the second highest amount of nitrosamines to the diet. According to results from the same German study, approximately 0.21 μg of nitrosamines is derived per person per day from this source (approximately 16% of the total dietary intake of nitrosamines).

Average relative exposures to nitrosamines by the U.S. population have been estimated by the committee for a variety of sources. With the exception of occupational exposures, which were not considered in the calculations, cigarette smoking contributes the greatest amount to total daily nitrosamine intake. Among the other sources considered (i.e., automobile interiors, beer, cosmetics, cooked bacon, and Scotch whiskey), Scotch whiskey contributed the lowest daily intake and the other four sources contributed intermediate amounts ranging from 0.16 to 0.97 $\mu\text{g}/\text{person}/\text{day}$.

The committee makes the following recommendations based on the data reviewed in this chapter:

1. The committee recommends that methods for the analysis of nonvolatile N-nitroso compounds be further developed and applied so that the total burden of these chemicals in the human population can be assessed.

2. The committee suggests that the origins of exposure to N-nitroso compounds in the various environmental media be determined and that methods to eliminate or reduce the production of these contaminants be developed. In this regard, the committee would

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CHAPTER 8

METABOLISM AND PHARMACOKINETICS OF NITRATE, NITRITE, AND N-NITROSO COMPOUNDS

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METABOLISM AND PHARMACOKINETICS OF NITRATE, NITRITE, AND N-NITROSO COMPOUNDS

This chapter describes metabolic pathways that are pertinent to evaluating the contribution of nitrate, nitrite, and N-nitroso compounds to adverse health effects, which are reviewed in Chapter 9. The discussions focus on the metabolism of nitrate and nitrite (especially the reduction of nitrate to nitrite in the saliva) and the metabolism of N-nitroso compounds (especially those reactions that are important in the carcinogenic effect of these compounds). In addition, evidence for endogenous synthesis of nitrate, nitrite, and N-nitroso compounds is also reviewed. The committee then uses data from a study on endogenous synthesis of N-nitroso compounds in humans to estimate possible exposures from this source in several different population groups. These estimates of endogenous exposures are compared to estimates of exogenous exposures to N-nitroso compounds developed in Chapter 7.

METABOLISM OF NITRATE AND NITRITE

As pointed out in Chapter 5, the exposure of humans to nitrate and nitrite varies from individual to individual and is dependent on place of residence (which determines water supply and presence or absence of smog) and lifestyle (e.g., food consumption and smoking habits). Moreover, exposure to these two ions from exogenous sources varies considerably over time for a given individual who lives in one location and maintains reasonably constant dietary habits. This occurs because intakes of nitrate and nitrite generally fluctuate, occurring in "pulses"; for example, they are higher following ingestion of a nitrate-containing vegetable or a nitrite-containing cured meat product. Thus, average exposures will not reflect individual fluctuations and peak intakes. Similarly, the discussion of the metabolism of these chemicals is, of necessity, based on generalizations and will not reflect individual variation due to differences in physiology, age, and overall health.

Oral Cavity

Transport of Nitrate to Saliva. Ingested nitrate is absorbed from the gastrointestinal tract into the bloodstream, which carries it to the salivary glands (Spiegelhalter et al., 1976). Because nitrate, thiocyanate, and iodide appear to share a common active transport

reasonable to assume that concentrations of iodide and thiocyanate in the blood could inhibit transport of nitrate when levels of nitrate in the blood are low (Hartman, 1981). In fact, Spiegelhalter et al. (1976) found that a corresponding peak in salivary nitrate concentration was not consistently detected until a certain threshold of ingested nitrate had been surpassed. The proposed threshold dose of nitrate was approximately 54 mg of ingested nitrate (Figure 8-1). These investigators also found that at oral doses of nitrate above the threshold, roughly 25% of the ingested nitrate was recirculated into the saliva (Figure 8-1).

Studies in laboratory animals have shown a wide variation in the efficiency of salivary transport of nitrate, thiocyanate, and iodide ions (Brown-Grant, 1961; Burgen and Emmelin, 1961). These differences must be considered when extrapolating data from experiments in animals to humans.

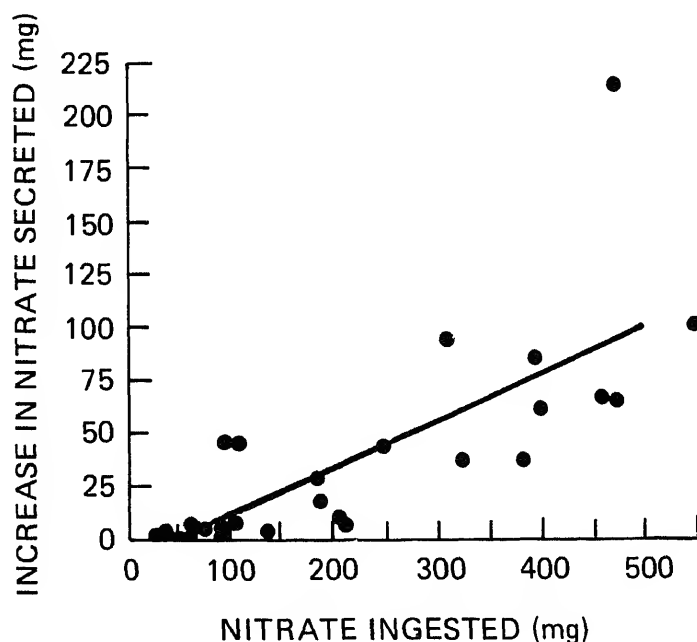


FIGURE 8-1. Ingestion of nitrate and salivary secretions of nitrate.

may also contain appreciable concentrations of nitrate (Turek et al., 1980a,b), which may result from the absence of thiocyanate competition.

Bacterial Reduction of Nitrate to Nitrite. Tannenbaum et al. (1974) reported that levels of nitrite in the saliva of humans usually ranged from 6 to 10 mg/liter and that these levels did not change significantly following the consumption of a meal. More recently, however, Spiegelhalder et al. (1976) found that salivary nitrite concentration was directly proportional to the amount of nitrate ingested. For example, when vegetables with a high nitrate content were consumed, the nitrite concentration in saliva showed a corresponding peak that was approximately 5% of the amount of nitrate ingested. Tannenbaum et al. (1976) reinvestigated their earlier findings and found that high intakes of nitrate produced large increases in salivary nitrite concentrations. They decided that their previous conclusions (Tannenbaum et al., 1974) were oversimplified and may have been due to the fact that the diets of the subjects studied did not contain nitrate-rich food or water.

Measurements of nitrate and nitrite concentrations in the saliva of humans following the ingestion of high-nitrate-containing vegetables or sodium nitrate have demonstrated that nitrate is reduced to nitrite by bacteria present in human saliva and that some of these bacteria possessed nitrate reductase activity in vivo (Ishiwata et al., 1975a,b) and in vitro (Ishiwata et al., 1975c).

The nitrate reductase enzyme is a non-heme iron protein that contains various subunits and molybdenum (Bryan, 1981; Payne, 1981; Stouthamer, 1976; Yordy and Ruoff, 1981). It has been studied in several microorganisms including Escherichia coli, Klebsiella aerogenes, Proteus mirabilis, Bacillus stearothermophilus, Bacillus licheniformis, Haemophilus parainfluenzae, and Pseudomonas aeruginosa. The saliva of humans contains bacteria that are capable of reducing nitrate to nitrite. These bacteria belong to several genera, including Staphylococcus, Veillonella, Corynebacterium, and Fusobacterium (Tannenbaum et al., 1974). In one study, Brown et al. (1975) reported that the median counts of these bacteria in the stimulated saliva of six adults were as follows (all units are $\times 10^6/\text{ml}$): Veillonella = 4, Fusobacterium = 3, and Staphylococcus = 0.0035.

However, there is considerable variation in the amounts and kinds of oral bacteria in healthy individuals (Kraus and Gaston, 1956; Wilson and Miles, 1964). In many cases, humans appear to have the propensity for acquiring and maintaining certain microbial strains or mixtures of strains, and each strain has its own novel

metabolic spectrum and capacity. For example, some bacteria, such as Bacillus subtilis, competently reduce nitrate to nitrite but cannot utilize nitrite further, whereas other bacteria can contribute both to the formation and the degradation of nitrite (Selenka, 1970). Thus, interactions in a mixed bacterial flora can be quite complex.

The amount of nitrite produced from ingested nitrate in the saliva is determined not only by the number and kinds of microorganisms containing the nitrate reductase enzyme, but also by a number of other factors such as the amount of oxygen available to the bacteria. For example, when Klebsiella is shifted from anaerobic to aerobic conditions, there is a cessation in both the production of nitrite and the synthesis of nitrate reductase (Stouthamer, 1976). However, it has been shown that salivary reduction of nitrate to nitrite can occur under both aerobic and anaerobic conditions.

Nitrate reduction can also be enhanced or inhibited by other factors, such as salivary pH. The optimum pH for the conversion is 6.0 to 6.4. Nitrate reductase activity is often "inducible;" that is, it is present only after bacteria have been exposed to elevated nitrate concentrations for a certain period. This has led to the hypothesis that a continually elevated nitrate concentration in the saliva may select for bacteria capable of nitrate reduction (Hartman, 1981). Finally, compounds readily metabolized by bacteria are essential for maximal nitrate reductase activity once the enzyme complex is formed, although the optimal carbon source may vary among bacterial species (Stouthamer, 1976; Wallis, 1913). Thus, several factors in addition to the bacterial content of the oral cavity exert important influences on the rates at which nitrate is reduced to nitrite in the saliva.

Nitrate and Nitrite Content of Saliva. Persons on low-nitrate diets who are "pulsed" with significant amounts of nitrate exhibit a characteristic level of nitrite production, which amounts to roughly 5% of nitrate ingested (Spiegelhalder et al., 1976; Figure 8-2), that is, the nitrite concentration (product) is roughly proportional to the nitrate concentration (substrate) of the saliva (Spiegelhalder et al., 1976), and approximately 20% of the salivary nitrate is reduced to nitrite (Figure 8-3). Stephany and Schuller (1980) have also reported that the amount of ingested nitrate is directly proportional to salivary nitrite concentration. They calculated that the average conversion of nitrate to nitrite in the saliva of the average healthy adult is approximately 6.3% mol % (i.e. approximately 5% by weight) of total dietary nitrate intake during a 24-hour period.

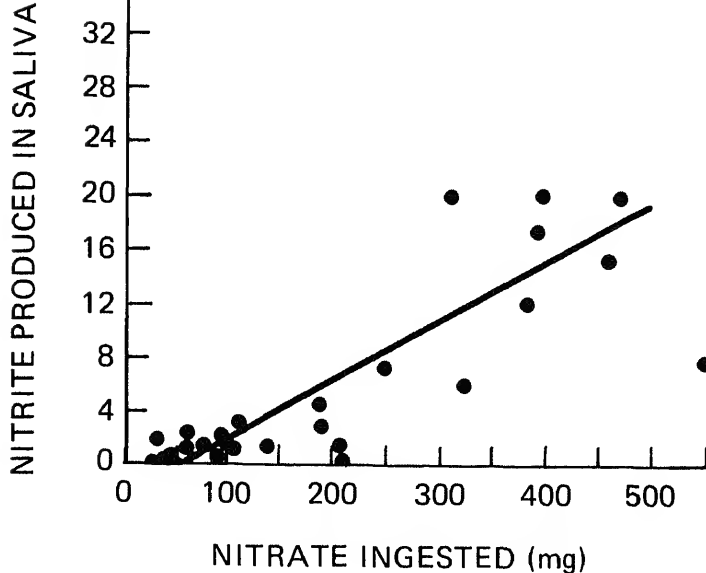


FIGURE 8-2. Nitrite in the saliva of persons on low-nitrate diets. These subjects were "pulsed" with different oral doses of nitrate. From Spiegelhalter et al., 1976.

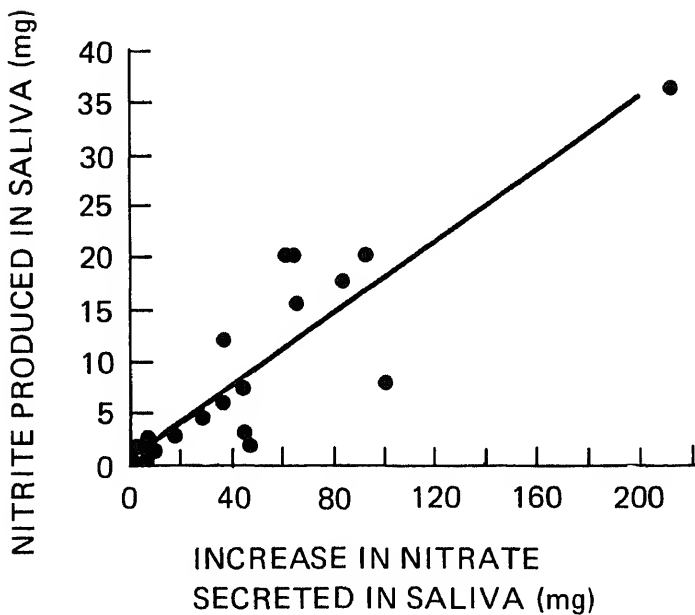


FIGURE 8-3. Relationship between the increase of salivary nitrate

Sources of Nitrate. All ingested nitrate and nitrite ions enter the stomach, except for a small amount that is metabolized and assimilated by bacteria in the oral cavity and the esophagus. To this exogenous nitrate and nitrite load are added nitrate derived from recirculation of ingested nitrate through salivary excretion and nitrite produced by bacterial reduction of part of this nitrate in the oral cavity and the esophagus.

To these two sources of gastric nitrate should be added a third source: nitrate that is secreted directly into the stomach. Two physiologically different mechanisms have been proposed to explain how direct secretion may occur: (a) anion secretion (a nitrate ion displaces a chloride ion) and (b) active transport of nitrate into neutral and alkaline gastric secretions by a saturable carrier complex, in which iodide and thiocyanate ions compete for transportation. These two proposed mechanisms are based on studies of the gastric transport of thiocyanate (Logothetopoulos and Myant, 1956) and iodide (Davenport, 1943; Halimi and Stuelke, 1959; Honour et al., 1952; Howell and Van Middlesworth, 1956; Logothetopoulos and Myant, 1956; Mason and Bloch, 1950; Myant et al., 1950; Oeff et al., 1955; Ruegamer, 1963; Schiff et al., 1947).

Normally, anion secretion will not be an effective means of introducing nitrate into the lumen of the stomach since the molar concentration of the chloride ion in plasma normally exceeds the molar concentration of the nitrate ion by a factor of perhaps 1,000. Persons sustained on diets containing high levels of nitrate and those with impaired kidney function can accumulate up to 19 mM nitrate (Keith et al., 1930). However, this is still much less than the normal plasma chloride level of approximately 103 mM (Diem and Lentner, 1970).

On the other hand, active transport of nitrate into the gastric lumen could be an important physiological process, although it has not been considered thus far (Hartman, 1981). The above-cited studies of iodide transport indicate that active transport occurs throughout the stomach and is independent of gastric pH. Furthermore, the gastric transport of iodide in the rat is not an intrinsically regulated process. The amount transported can reach saturation and is dependent upon plasma iodide concentration (Halimi and Stuelke, 1959). In this case, transport of nitrate directly into gastric juices can account for the presence of appreciable quantities of gastric nitrate within minutes after the ion is injected intravenously (Balish et al., 1981; Witter et al., 1979a). The ratio of nitrate concentrations in the gastric juice compared to those in the plasma can reach 20:1 in rats ligated at the pyloric sphincter and given nitrate intravenously. Active transport appears

had been actively transported into the stomach from the plasma.

Source of Nitrite. In order to estimate the total nitrite load in the upper gastrointestinal tract of adults with normal gastric acidity, one must consider both ingested nitrite and nitrite produced from salivary nitrate, which is recirculated following ingestion. Table 8-1 lists the estimates developed in Chapter 5 for the average daily nitrate and nitrite ingestion categorized by source for adults (see Tables 5-20 and 5-21). The amount of nitrite formed in the saliva from ingested nitrate is given in column 3 of Table 8-1. In calculating these figures, the committee assumed that approximately 5% (6.3 mol %) of the ingested nitrate is converted to nitrite (see footnote c). Total nitrite and the percent of total nitrite derived from each category are also given in the table.

According to these estimates, which are based on average intakes of nitrate and nitrite, the main dietary contributors to gastric nitrite load are vegetables (~72%), cured meats (~9%), and baked goods and cereals (~7%). However, none of these estimates include calculations of nitrate and nitrite loss due to bacterial or mammalian assimilation, nor do they consider the effect of possible endogenous synthesis of nitrate or nitrite (a possibility discussed later in this chapter).

Another source of nitrate in the upper gastrointestinal tract is reduction of nitrate by bacteria present in the achlorhydric stomach. Survival and proliferation of microorganisms in the human gastric lumen is dependent, among other things, on the pH (Bartle and Harkins, 1925; Franklin and Skoryna, 1971; Giannella et al., 1972; Knott, 1927; Lampé and Strassburger, 1943). Generally, a pH of approximately 4.5 to 5.0 is necessary to prevent substantial bacterial invasion by a mixed bacterial population that, in adult humans, includes a spectrum of nasopharyngeal and salivary bacteria (Bartholomew et al., 1980; Drasar et al., 1969; Franklin and Skoryna, 1971; Hawksworth et al., 1975; Lampé and Strassburger, 1943; Sander and Seif, 1969; Schweinsberg et al., 1975).

Gastric anacidity characterized by pH values often rising above pH 5 is relatively commonplace among the general population. This condition occurs more frequently and to a greater degree in older populations and may involve a majority of individuals over 70 years of age (Hartman, 1981). Even in persons with "normal" gastric acidity, the pH may occasionally rise sufficiently to permit bacterial

Exposure, mg/Person/Day^a

Source	Dietary Nitrite ^b	Dietary Nitrate ^b	Salivary Nitrite ^c	Total Nitrite in Upper Gastrointestinal Tract	Percent Contribution of Nitrite from Each Source (Approximate)
Cured meats	0.30	1.2	0.06	0.36	9
Fresh meats	0.06	0.6	0.03	0.09	2
Vegetables	0.12	65	3.0	3.1	72
Fruits, juices	0.01	4.3	0.20	0.21	5
Baked goods and cereals	0.26	1.2	0.06	0.32	7
Milk and milk products	0.01	0.2	0.01	0.02	< 1
Water	0.01	2	0.09	0.10	2
TOTAL	0.77	75	3.5	4.2	

^aWhere appropriate, values have been rounded off to two significant figures.

^bData from Tables 5-20 and 5-21.

^cCalculated by multiplying intake of nitrate by 6.3 mol % (0.0467) (Spiegelhalder et al., 1976; Stephany and Schuller, 1980). See text for discussion of conversion of nitrate to nitrite in saliva.

invasion (Atkinson and Henley, 1955; Franklin and Skoryna, 1971), thereby providing an opportunity for extensive nitrite formation (Schweinsberg et al., 1975).

Ruddell et al. (1980) observed an elevated gastric pH in patients with ulcers who had been treated with cimetidine. They also found an increase in gastric bacterial flora, which included large numbers of fecal organisms. Such bacteria are also present in the stomach of achlorhydric patients who are at increased risk for gastric cancer.

Most infants normally possess an elevated gastric pH, but it is especially high in infants fed cow's milk (Marriott and Davidson, 1923; Oliver and Wilkinson, 1933; Vanzant et al., 1932). The

on a mg/kg body weight basis (Chapter 5), and it has been well documented that early exposure to environmental substances is an important determinant of risk of gastric cancer much later in life (Hartman, 1981).

Gastric nitrite concentration appears to be influenced by the total content of nitrate-reducing bacteria rather than by a preponderance of any specific organism (Bartholomew *et al.*, 1980). The rate of nitrate reduction by bacteria in the stomach is also influenced by metabolizable carbon sources (Hartman, 1981).

As shown in Table 8-2, which presents a hypothetical example of the possible nitrite load in the stomach under conditions of hypoacid and mixed bacterial infection, approximately half of the gastric nitrate may be reduced to nitrite in certain individuals. Thus, the bulk of gastric nitrite exposure in achlorhydric individuals may stem from the reduction of nitrate within the stomach itself.

TABLE 8-2

Hypothetical Example of Gastric Nitrite Load in Person with Gastric Hypoacidity and Mixed Bacterial Infection

<u>Source</u>	<u>Exposure, mg/Person/Day</u>		<u>Percent Total Gastric Nitrite</u>
	<u>Nitrate</u>	<u>Nitrite</u>	
Ingested ^a	75	0.77	1
Recirculated Salivary ^b	19	4	7
Gastric ^c	19	54 ^d	92
TOTAL	109 ^e	59	--

^aNitrate and nitrite ingested for average U.S. population (Tables 5-20 and 5-21).

^bRecirculated nitrate equals 25% ingested nitrate (Speigelhalter *et al.*, 1976).

^cAssumes recirculation of nitrate to stomach via active transport equals salivary recirculation of 25%.

^dAssumes 50% of the total gastric nitrate is reduced to nitrite.

^eOver a 24-hour period, 75 mg ingested nitrate reaches stomach, plus

The uptake of nitrate from the gastrointestinal lumen occurs primarily in the small intestine and appears to be an active process -- probably sharing carriers that also mediate iodide transport. There is little or no uptake of nitrate from the stomach. For example, as mentioned previously, nitrate is retained in the stomach of animals ligated at the pyloric sphincter (Witter et al., 1979b). In humans, most of the gastric nitrate passes into the small intestine (Witter et al., 1979b); however, small amounts of nitrate could penetrate through the gastric mucosa if nitrate behaves like iodide (Brown-Grant, 1961; Halmi, 1964).

In contrast, the transport of nitrate across the wall of the proximal small intestine is rapid and reasonably complete, at least in laboratory animals (Balish et al., 1981; Hawksworth and Hill, 1971; Ishiwata et al., 1977; Keith et al., 1930; Witter and Balish, 1979; Witter et al., 1979a). Studies in laboratory animals have also revealed that rats can excrete nitrate into the lumen of the mid-portion of the small intestine (Balish et al., 1981; Witter and Balish, 1979; Witter et al., 1979a). Therefore, the observation that nitrate is present in the lower small intestine of rats is no assurance that there has not been essentially complete absorption in the proximal small intestine. This ability to excrete nitrate and iodide (Acland and Illman, 1959) into the small intestine is unique to rats and does not apply to humans or to other animals tested (Brown-Grant, 1961) -- a fact that must be considered when trying to extrapolate the results of studies on nitrate metabolism in rats to humans.

The penetration of the gastrointestinal mucosal barriers by nitrite has not been carefully measured at concentrations normally ingested. There have been reports that nitrite may pass through the mucosa following erosion and hemorrhage in cases of intense gastritis (Gwatkin and Plummer, 1946; Sollman, 1957). In humans, massive lethal doses of oral nitrite are predominantly retained in the stomach and its contents; very small amounts reach the intestine, and much less reaches the liver, kidney, or urine (Naidu and Venkatrao, 1945). Much lower levels of ingested nitrite can prolong the emptying time of the normal human stomach (Sleeth and Van Liere, 1941), and gastric paralysis appears almost complete at lethal doses of nitrite (Naidu and Venkatrao, 1945).

The rate at which nitrite disappears from the stomachs of laboratory animals is greater than would be expected from the kinetics of gastric emptying (Friedman et al., 1972; Ishiwata et al., 1977; Mirvish et al., 1975), and it occurs in mice ligated at the gastroduodenal junction (Friedman et al., 1972). Some of this nitrite loss can be accounted for by chemical oxidation of nitrite

be detected. The disappearance of gastric nitrite in vivo in conventional animals also follows second-order kinetics, indicating involvement of dinitrogen trioxide (Friedman et al., 1972). In this process, there is active gastric absorption of dinitrogen trioxide (Friedman et al., 1972), but much, if not all, of the loss could be equally due to chemical reactivity of dinitrogen trioxide with components of gastric chyme, such as urea and mucins, as well as with surfaces of gastric epithelial cells. The importance of these reactions in the in vivo formation of N-nitroso compounds will be discussed later in this chapter.

Some nitrite is carried into the small intestine where chemical oxidation to nitrate may continue, but again little or no absorption of nitrite is evident (Witter and Balish, 1979).

Transport and Chemical Reactions in the Blood

No appreciable reduction of nitrate to nitrite has been observed in the mammalian circulatory system (Parks et al., 1981). Rather, plasma nitrate appears to circulate and to participate in rapid dissemination processes that often mimic the flow of iodide. Relatively low plasma levels of nitrate are maintained by a presumably reversible, rapid dissemination in tissues that occurs within minutes. This fast-equilibrating nitrate compartment represents approximately 28% of the body weight of dogs (Greene and Hiatt, 1954) and is the same relative size as the rapidly equilibrating iodide compartment of humans (Myant et al., 1950). Thus, pulses of nitrate introduced orally or administered intravenously are rapidly disseminated in the mammal, preventing accumulation in the blood even before nitrate reaches the urine (Balish et al., 1981; Parks et al., 1981; Witter and Balish, 1979; Witter et al., 1979a,b,c).

Nitrate is also removed from plasma in the kidneys, ending up largely as nitrate in urine. Thus, urinary excretion is another important mechanism for the maintenance of low plasma nitrate levels.

Nitrite reaching the circulatory system by slow diffusion or through lesions in mucosal barriers rapidly reacts with oxyhemoglobin to form methemoglobin (Chapter 9). The rate at which nitrite reacts with oxyhemoglobin varies widely among species (Parks et al., 1981; Rath and Krantz, 1942; Smith and Beutler, 1966). Thus, care must be used when extrapolating to humans data obtained from experiments in animals. For example, the formation of methemoglobin in pigs is much slower (Smith et al., 1978) than in other species examined (Smith and Beutler, 1966). If nitrite does not react immediately with hemoglobin in the maternal circulation, an effective portion of

transformation of fetal cells (Inui et al., 1979a,b), as observed in laboratory animals.

In all the species studied, the action of enzymes in the red blood cell restores hemoglobin function and releases free nitrate in the process (Smith and Beutler, 1966).

Excretion of Nitrate in Urine

In contrast to the rapid oxidation of ingested nitrite to nitrate, the dissemination of nitrate into the major tissue compartments, and recirculation of nitrate by active transport systems, all of which occur within minutes, there may be more slowly operating processes that affect nitrate flow, for example, in more slowly equilibrating nitrate and iodide tissue compartments such as the cerebrospinal fluid (Hiatt, 1940; Myant et al., 1950).

In the rat, additional nitrate is withdrawn from the system by its active secretion into the mid-portion of the small intestine; failure of efficient transport from the lower gastrointestinal tract then allows excretion in feces or assimilation by bacteria in the large bowel (Balish et al., 1981; Green et al., 1981; Witter and Balish, 1979; Witter et al., 1979a). In germ-free rats, approximately 16% of ingested nitrate is excreted in the feces (Green et al., 1981). However, as pointed out above, active transport of nitrate from the circulatory system into the intestine is unlikely to occur in the human (Brown-Grant, 1961). Essentially all of the nitrate ingested by humans, especially at higher levels, appears in the urine (Hawsworth et al., 1975).

The effect of increased and decreased nitrate ingestion on the urinary excretion of nitrate has been studied in humans (Hill, 1979; Keith et al., 1930; Mitchell et al., 1916; Tannenbaum et al., 1978) and in laboratory animals (Balish et al., 1981; Green et al., 1981; Keith et al., 1930). Urinary excretion of nitrate after ingestion of large oral doses of nitrate has also been studied in humans (Hill, 1979; Ishiwata et al., 1978) and in laboratory animals (Hawsworth and Hill, 1971; Hill, 1979; Wang et al., 1981). In humans, total urinary excretion of nitrate takes several days (Hill, 1979), although the bulk is eliminated in a shorter period. Excretion of nitrate from humans is reported to follow first-order kinetics, having an average elimination half-life of 5 hours (Green et al., 1981).

Nitrite is not a normal constituent of urine, but tens of milligrams of nitrite can be formed in urine daily by bacterial action during urinary tract infections (Polach et al., 1976).

substrate (nitrate) available for reduction and, thus, to the amount of nitrate and nitrite ingested by the individual. Implications that nitrate reduction is involved in the induction of bladder cancer' are discussed later in this chapter.

Endogenous Synthesis of Nitrate and Nitrite

In addition to acquiring nitrate and nitrite from exogenous sources, endogenous formation of these ions may also occur. Two hypotheses have been advanced to explain the mechanisms of endogenous synthesis -- heterotrophic bacterial nitrification and synthesis in mammalian tissues.

Heterotrophic Bacterial Nitrification. Tannenbaum and coworkers (1978) described the endogenous synthesis of nitrite and nitrate in humans. Using a modified Griess procedure, they measured components of urine and the diet for nitrate and nitrite content in order to conduct nitrate balance studies. Their results indicated that there were wide fluctuations in urinary nitrate on a day-to-day basis and that urinary excretion of nitrate greatly exceeded the intake of nitrate. Similar observations were reported by Mitchell et al. (1916). In addition, analysis of fecal samples conducted by Tannenbaum et al. (1978) showed the presence of both nitrate and nitrite, whereas ileostomy effluents contained nitrite, but no detectable nitrate.

In order to explain these puzzling results, Tannenbaum et al. (1978) concluded that "heterotrophic nitrification of ammonia or organic nitrogen compounds takes place in the upper, aerobic portion of the intestine" in much the same way that such processes occur in other ecosystems such as those of sewage, soil, lakes, and rivers. Continuing this rationale, they postulated that the nitrification process could lead to the formation of nitrate/nitrite in the more anaerobic large intestine and that N-nitroso compounds may be synthesized in the acidic environment of the cecum and colon.

Another explanation of the findings reported by Tannenbaum and coworkers was offered by Witter et al. (1979b), who studied the distribution of $^{13}\text{NO}_3^-$ in humans and rats. Their results indicated that approximately 25% of gavaged nitrate and nitrite could pass directly into the lower intestinal tract and could account for the nitrate and nitrite in fecal samples and the nitrite in ileostomy samples detected by Tannenbaum et al. (1978). Since humans and rats have the capability of temporarily storing nitrate and nitrite in the extravascular spaces of the body (as measured by studies with labeled nitrate), a slow washing of nitrate from the body could

concentrations in urine and saliva 2 to 5 hours after administration and a clearance phase with a half-life of approximately 8 hours. Furthermore, if no additional nitrate enters the body, the nitrate could be cleared within 48 hours, independent of the pool concentration, since the clearance of nitrate follows first-order kinetics.

Witter et al. (1979c) noted that nitrate and nitrite ingested in foods can be difficult to detect with the Griess test, bringing into question the accuracy of dietary levels measured by Tannenbaum et al. (1978). These workers also pointed out that Tannenbaum et al. (1978) had not considered the contribution of endogenous nitrate from nitrogen oxides. Witter et al. (1979c) calculated that as much as 500 mg of nitrate could be formed per month if 6 liters of air containing 1 ppm ($\sim 1,880 \mu\text{g}/\text{m}^3$) nitrogen dioxide are inhaled per minute.

Witter et al. (1979b,c) questioned the heterotrophic nitrification hypothesis on the basis that the intestinal tract is probably predominantly anaerobic. Furthermore, oxidation of ammonia to nitrate would require vast numbers of organisms, and the upper intestine contains few bacteria. Since heterotrophic nitrification was expected to occur at this site, it appears that the conditions might not be suitable for such a process. An alternative explanation for ileal nitrite levels reported by Tannenbaum et al. (1978) would be bacterial reduction of nitrate to nitrite rather than oxidation of ammonia to nitrite (Witter et al., 1979b,c).

Mammalian Synthesis. Nitrate balance studies were conducted by Tannenbaum's group (Green et al., 1981) in germ-free and conventional Sprague-Dawley rats to test the hypothesis that nitrate is synthesized endogenously by heterotrophic bacterial nitrification. The basal diet of these animals was supplemented with $\text{Na}^{15}\text{NO}_3$ so that the pools of nitrate could be distinguished using mass spectrometric measurements of the ^{15}N and ^{14}N abundance ratios. Results of analysis of nitrate in the diet and in the urine indicated that at various levels of nitrate ingestion, the urinary output of sodium nitrate exceeded that of the dietary intake for both germ-free and conventional animals. Since both animal groups excreted similar levels of nitrate, the role of the bacterial flora in the synthesis of nitrate was eliminated. Thus, the authors postulated that mammalian synthesis, rather than their original suggestion of heterotrophic nitrification, was the most likely source of excess nitrate.

Witter et al. (1981) measured dietary and urinary nitrate by high performance liquid chromatography (HPLC) and found that the intestinal flora of conventional rats on a chow diet actually decreased the urinary excretion of nitrate, whereas there was an increase of nitrate

good (although variable) recoveries (approximately 70-80%), they found that nitrate and nitrite levels in feces and ileostomy fluid were much lower than those published by Tannenbaum et al. (1978). They concluded that the maximum fecal excretion of nitrate and nitrite is much lower than the normal dietary intake and that during in vitro incubations with added nitrite, no oxidation to nitrate occurred. Although there are many possible explanations why nitrite (or lower oxidation states of nitrogen) cannot be converted to nitrate by intestinal microorganisms, it appears that conditions in the lower gastrointestinal tract favor reduction of nitrate and nitrite (Saul et al., 1981). Therefore, heterotrophic nitrification appears to be an inadequate explanation of the results obtained in the nitrate balance studies of Tannenbaum et al. (1978), and mammalian synthesis of nitrate appears to be more plausible (Green et al., 1981; Parks et al., 1981).

METABOLISM OF N-NITROSO COMPOUNDS

The metabolism of nitrosamines has been exhaustively reviewed (Magee, 1980; Magee and Barnes, 1967; Magee et al., 1976; Montesano and Bartsch, 1976; Pegg, 1980). It is not the purpose of this report to recapitulate all the published data but, rather, to summarize the general principles of nitrosamine metabolism, with emphasis on recent studies, in order to aid in the evaluation of the potential risk of these carcinogens to humans.

Of the large number of nitrosamines that have been found to be carcinogenic in laboratory animals, only a few, especially nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA), have been studied in any great detail. However, alkylation, mutagenesis, teratogenesis, and carcinogenesis studies with a number of nitrosamides can also provide insight into the potential consequences of exposure to nitrosamines since the alkylating species, which are considered to cause the pathologic changes, are similar for nitrosamines and nitrosamides. The major difference is that nitrosamides, in contrast to nitrosamines, are quite unstable and do not require metabolic activation.

Pharmacokinetics

As a result of his early biochemical studies, Magee concluded that intravenously administered NDMA rapidly equilibrates throughout the body of rats (Magee, 1956). This conclusion was confirmed and extended by an autoradiographic study of transected mice. That study was designed to determine the distribution of radioactivity in the organs of mice that had received intravenous injections of ¹⁴C-labeled

the in vivo metabolism of NDMA. In addition, the unmetabolized, volatile NDMA was evaporated from the transected tissue block by lyophilization. Thus, the exposure of x-ray film to the lyophilized block revealed only the bound (nonvolatile) products of NDMA metabolism, whereas exposure of the film to the tissue at -80°C revealed both metabolized (nonvolatile) and unmetabolized (volatile) NDMA.

Autoradiography of the pretreated mice at -80°C demonstrated that radioactivity was uniformly distributed throughout the organs after 30 minutes and that there was no radioactivity in the tissues where evaporation had occurred. This confirmed that unmetabolized, volatile NDMA was distributed equally throughout the body tissues. In contrast, in animals that had not been pretreated, selective labeling of certain organs, including liver, pancreas, salivary gland, intestinal mucosa, and bone marrow, was evident after 30 minutes. The investigators also demonstrated that as soon as 1 minute after injection of the ^{14}C -labeled NDMA, radioactivity was concentrated mainly in the liver and kidney and there was some diffuse background activity in other tissues in samples maintained at -80°C . Furthermore, even after the transected tissue blocks were freeze-dried and evaporated, the radioactivity persisted in the liver and renal cortex; however, the diffuse radioactivity in other tissues had disappeared.

These results add strong support to the notion that the striking organotropic, toxic, and carcinogenic effects of nitrosamines are not due to the preferential uptake of the unmetabolized carcinogen but, rather, to preferential metabolism by specific organs. Furthermore, since the organ distribution of ^{14}C at 30 minutes after injection of ^{14}C -labeled NDMA resembled that observed at 30 minutes following injection of ^{14}C -labeled formaldehyde (with the exception of the liver, where concentrations were much higher in the NDMA-injected mice), the degraded products of ^{14}C -labeled NDMA are apparently incorporated into the 1-carbon pool of protein-synthesizing or replicating cells. In both instances, radioactivity between 30 minutes and 24 hours after injection was high in organs that had the highest rates of cell replication, including bone marrow and intestinal mucosa.

In contrast to these findings from intravenous administration of NDMA, there is good evidence that certain ingested nitrosamines and those formed in vivo in the upper gastrointestinal tract are absorbed rapidly from the duodenum (Hashimoto et al., 1976) and subsequently carried by the portal circulation to the abdominal viscera. When low doses of nitrosamines are ingested, the liver appears to behave as a "first line of defense" by markedly reducing the concentration of nitrosamines in the systemic circulation. This inference is drawn from a comparison of the degree of alkylation of kidney and liver DNA following oral or intravenous administration of NDMA (Page, 1980).

greater after oral doses than after intravenous administration. In one sense, this is reassuring, since O⁶-methylguanine excision and repair rates appear to be higher in the liver than in other organs of the rat (Brash and Hart, 1978). On the other hand, this preferential hepatic metabolism following oral intake suggests that the total body burden of nitrosamines may be higher than estimates based on peripheral venous blood concentrations (Pegg, 1980).

Activation and Deactivation

In common with a broad range of other carcinogenic agents (Miller and Miller, 1971b), the ultimate toxic and carcinogenic species resulting from the metabolism of nitrosamines are electrophiles, which are capable of covalently reacting with cellular macromolecules including DNA, RNA, and protein (Pegg, 1977). However, although tumor induction occurs only in organs in which alkylation takes place (Magee and Barnes, 1967; Magee et al., 1976; Montesano and Bartsch, 1976), the nature of the alkylating molecule is not known for any given nitrosamine. The primary reaction required for the activation of nitrosamines is considered by most investigators to involve an enzyme-mediated α -hydroxylation requiring reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen. β -Hydroxylation of nitrosodialkylamines yielding a nitrosomethylalkylamine may also occur prior to α -hydroxylation and may account for methylation by longer chain nitrosodialkylamines (Krüger, 1972; Lawson et al., 1981a,b).

Recent studies with nitrosomethyl(acetoxymethyl)amine strongly support the role of α -hydroxylation (Pegg, 1980). After intravenous administration of this nitrosamine, the spectrum of DNA-alkylated products is identical to that observed after NDMA activation (Kleihues et al., 1979). Since neoplasms are mainly induced close to the sites of injection of nitrosomethyl(acetoxymethyl)amine (Berman et al., 1979; Habs et al., 1978; Kleihues et al., 1979; Wiessler and Schmähl, 1976), and activation is solely dependent on the activity of a ubiquitous esterase, it appears that the only requisite for activation of the carcinogen is generation of an α -hydroxylated product, which then undergoes spontaneous decomposition and leads to alkylation. Mochizuki et al. (1980) recently succeeded in synthesizing the α -hydroxy derivatives of nitrosodialkylamines and found that they were not as unstable as previously believed. Furthermore, these derivatives were recently shown to be mutagenic (Maekawa et al., 1981). Formaldehyde, generated in the course of subsequent oxidative demethylation, is entirely degraded to carbon dioxide. However, it may take a long time for carbon dioxide to form, especially since

to validate the postulated pathway. On the other hand, one would expect near quantitative recovery of nitrogen. In fact, this has been documented in at least two different laboratories (Magee, 1980; Milstein and Guttenplan, 1979).

Speculations concerning the intermediate steps in nitrosamine metabolism (Heath, 1962; Hultin *et al.*, 1960) continue to generate a great deal of research. Rose (1958) originally suggested that the major toxic derivative of NDMA metabolism was diazomethane. However, this possibility was subsequently ruled out by experiments in which fully deuterated NDMA was used (Lijinsky *et al.*, 1968). Since the alkylated hepatic nucleic acid derivatives contained three deuterium atoms, they could not possibly have arisen from diazomethane. Therefore, by a process of exclusion, it appears that either a methyl diazohydroxide ion or a methyl diazonium ion reacts with cell macromolecules. This reaction is believed to be involved in the process leading to cancer.

Czygan *et al.* (1973) discovered that the mutagenic action of NDEA was dependent on oxidative demethylation -- the result of a cytochrome-P450 metabolizing system. Therefore, it seems likely that the oxidative dealkylation necessary for the toxic and carcinogenic action of the nitrosamines is similarly dependent on cytochrome P450. However, another pathway, possibly dependent on amine oxidase, may also be involved (Lake *et al.*, 1975, 1976; Phillips *et al.*, 1977), since a number of anomalous aspects of the metabolism of NDMA cannot be easily explained by the dependency on a cytochrome-P450 system. These unexplained observations include inhibition rather than induction of metabolism by such classical inducers as phenobarbitone, polycyclic hydrocarbons, and polychlorinated biphenyls (Arcos *et al.*, 1975, 1977); atypical spectra when bound to cytochrome P450; weak inhibition by SKF 525; and stimulation of demethylation by metyrapone (Lake *et al.*, 1976). A number of high and low K_m dealkylating enzymes also have recently been described. Their identification has helped to explain some of the discrepancies in the literature, since "classical inducers" of cytochrome P450 generally inhibit the low K_m enzymes and stimulate the high K_m enzymes (Arcos *et al.*, 1975, 1977). It now appears that only the low K_m enzymes are of physiologic importance since the maximum nonlethal concentration of NDMA in body water is $10^{-3}M$ (Johansson and Tjälve, 1978). Accordingly, earlier studies of demethylase activity, in which high concentrations of substrate were used, do not reflect *in vivo* metabolism (Magee, 1980). The stimulation of NDMA demethylase by pretreatment with ethanol was described recently by Garro *et al.* (1981). This unconfirmed finding is potentially

The recognition that administration of nitrosamines results in the alkylation of DNA and the generation of at least 11 different nucleoside and alkyl-phosphotriester adducts (Pegg, 1977; Singer, 1979) is consistent with the somatic mutation hypothesis of carcinogenesis (Boveri, 1914; Burdette, 1955; Miller and Miller, 1971a). The major alkylated base produced by the administration of NDMA is 7-methylguanine (Magee and Farber, 1962). However, the formation of this adduct does not correlate with the relative carcinogenicity of NDMA, nitrosomethylurea, and methylmethane sulfate for the kidney (Swan and Magee, 1968). Furthermore, the degree of miscoding due to 7-methylguanine did not correlate with the degree of mutagenicity in bacteriophage following exposure to methylating and ethylating agents (Loveless and Hampton, 1969). Alkylation of DNA-derived guanine in the O⁶ position is a more likely cause of mispairing and mutation since this position is involved in base pairing.

In several classic studies, investigators have found that the persistence of O⁶-methylguanine in rats, probably as a consequence of impaired excision repair, seemed to correlate with the distribution of neoplasms following administration of nitrosourea (Goth and Rajewsky, 1974a,b; Kleihues and Margison, 1974). However, tests in some other species have indicated that there are exceptions to this correlation. For example, the susceptibility to development of neoplasms in the brain and liver of two different strains of mice did not seem to depend only on the formation and persistence of O⁶-ethylguanine in DNA (Buecheler and Kleihues, 1977). Furthermore, O⁶-methylguanine persists in gerbil brains after treatment with nitrosomethylurea, even though these animals do not develop brain tumors (Kleihues *et al.*, 1976). Additional adducts such as O⁴-alkylthymine, N'-alkylureas, and N-3-alkylpyrimidines are also suspect (Kröger and Singer, 1979; Lawley, 1974, 1976; Singer, 1979). Alkylphosphotriesters may affect chromatin structure (Cooper and Itzhaki, 1975), but their prevalence suggests they are unlikely to be strong mutagens. In the future, quantitation of adduct formation will be aided by the continued development of more refined radioimmunoassay procedures (Poirier, 1981; Poirier and Yuspa, 1981). These methods may ultimately become valuable in the assessment of human risk since the covalent binding of carcinogenic compounds can be studied in organ cultures of tissues derived from humans at autopsy (Harris, 1981).

Finally, it appears advisable to maintain proper perspective concerning adduct formation, repair mechanisms, and cell replication in assessing risks for humans. Frei (1976) has aptly referred to a "contest or race" between DNA replication and DNA repair that is of fundamental importance in the carcinogenic process. According to

carcinogens than after exposure to high doses (Pegg and Balog, 1979). Second, when assessing the results of experiments in animals, one should bear in mind that repair mechanisms are generally more efficient in the human than in other species.

In Vivo Formation of N-Nitroso Compounds

Humans may be exposed to N-nitroso compounds formed endogenously from a variety of precursors. These precursors may be amines and other nitrosatable substances and nitrosating agents inhaled in air, ingested in food, or formed in vivo from more elementary precursors. Evidence for the endogenous formation of N-nitroso compounds has come primarily from studies in laboratory animals. In contrast, the data pertaining to endogenously formed nitrosamines in humans are poor. Much of the information is still being subjected to scientific scrutiny, primarily to assess the efficacy of the analytical methods that have been used to gather the data. However, an experimental methodology for evaluating in vivo nitrosation in humans designed by Ohshima and Bartsch (1981) seems to have overcome all the earlier deficiencies. Their technique is discussed below.

Experiments with animals. Carcinogenicity, mutagenicity, and acute toxicity assays have been used as endpoints to detect in vivo nitrosation. In addition, nitrosamines in biological samples have been measured following ingestion of nitrite and amines.

In 1969, Sander and Bürkle induced esophageal and hepatic tumors in rats by feeding them N-methylbenzamine or morpholine plus nitrite. Tumors characteristic of the corresponding N-nitroso compound were also induced in rats fed nitrite plus methylurea, ethylurea, 1,3-dimethylurea, 2-imidazolidone, and N-methylaniline. Lung adenomas were induced in mice by long-term administration of nitrite plus morpholine, piperazine, N-methylaniline, methylurea, and ethylurea, but not with dimethylamine (Greenblatt et al., 1971; Mirvish et al., 1972). In a study of mice fed 6 g of piperazine plus nitrite for 1 to 40 weeks, the number of lung adenomas per mouse was approximately proportional to the concentration of piperazine in the food and the square of the concentration of nitrite, which was added to the drinking water (Greenblatt and Mirvish, 1973). The tumors in these studies were presumed to be caused by the endogenous formation of nitrosamines.

Whong et al. (1979) detected in vivo nitrosation in mice and rats in experiments using an intrahepatic, host-mediated mutagenicity assay with Salmonella typhimurium as the detecting organism. The sensitivity of the assay for NDMA was 0.2 mg/kg in mice and

gavage (Asahina et al., 1971). Similarly, rats gavaged with aminopyrine plus nitrite produced liver necrosis (Lijinsky and Greenblatt, 1972). Further evidence that liver damage was due to the formation of NDMA was provided by the studies of Montesano and Magee (1971), who detected [7-¹⁴C]methylguanine in the nucleic acids of the stomach, intestines, and liver, and attributed its presence to the formation of [¹⁴C]nitrosomethylurea.

Chemical analysis has also been used to determine the extent of in vivo nitrosation. Sander et al. (1968) found up to 30% yields of nitrosodiphenylamine and 4% yields of nitrosomethylaniline after gavage of the corresponding amines plus nitrite to rats (Sander and Schweinsberg, 1972). In vivo formation of nitrosoethylamine, nitrosopiperidine, and nitrosoproline was also confirmed by chemical analysis of the stomach contents of laboratory animals following administration of an appropriate amine plus nitrite (Alam et al., 1971a,b; Braunberg and Dailey, 1973).

Mirvish and Chu (1973) found nitrosomethylurea and nitrosoethylurea in the stomach of starved rats gavaged with urea plus nitrite. More recently, Mirvish et al. (1980) measured gastric nitrosomethylurea, formed from ³H-methylurea and nitrite. They found that the formation of nitrosourea was greater when rats were fed a semisynthetic diet with a low protein content than when they were fed a similar diet with a high protein content. This observation was correlated with the finding from an epidemiological study (Modan et al., 1974) that gastric cancer is associated with high-starch, low-protein diets (see Chapter 9).

In studies of the in vivo nitrosating potential of nitrogen oxides, Iqbal et al. (1980) gavaged mice with morpholine (2 mg/mouse) and exposed them to atmospheric nitrogen dioxide in concentrations up to 50 ppm for as long as 4 hours. They observed that nitrosomorpholine (NMOR) (up to 2.3 µg/mouse) was synthesized in vivo. Mirvish et al. (in press) reexamined the same system. They confirmed the results of Iqbal et al. using the same method, but did not obtain NMOR when a "stopping solution" containing ammonium sulfamate, ascorbic acid, and sulfuric acid was included in the preparation of the homogenate. This indicated that the nitrosamine formation occurred during the preparation of the whole-mouse homogenate in a methanol-water solution. This nitrosation may have been due to the in vivo formation of a nitrosating agent from the nitrogen dioxide -- an agent that did not nitrosate morpholine in vivo, but that did effect this reaction in the homogenate. The nature of this nitrosating agent, however, is currently unknown.

Lightsey (1981) reported that nitrogen dioxide reacts with unsaturated fats to produce nitrite. This latter reaction could also occur in vivo. The potential importance of these studies on the nitrosating potential of nitrogen oxides is indicated by the study of Van Stee et al. (1980), who reported a small but statistically significant increase in the yield of lung adenomas in mice exposed chronically to atmospheric nitrogen dioxide at 1 to 2 ppm and to morpholine in drinking water at 1 g/liter.

Detection of Endogenously Formed N-Nitroso Compounds in Humans.

Previous attempts to demonstrate possible in vivo formation of N-nitroso compounds in humans have focused on the detection of nitrosamines in feces, urine, or the blood. Although Wang et al. (1978) reported that relatively high levels of nitrosamines were present in feces of humans, further work has shown this result to be in error (Eisenbrand et al., 1981). These investigators discovered that a marker amine produced a nitrosamine when added to fresh feces; for example, morpholine produced NMOR. Thus, they suspected that the high levels detected in the earlier study could have resulted from the artifactual formation of nitrosamines during storage or during the analytical procedures.

Secondary amines, especially dimethylamine, piperidine, and pyrrolidine, are found in normal urine in levels as high as 6 mg/day (Drasar and Hill, 1974). These amines are presumably formed by bacterial action on breakdown products of food in the intestine (Asatoor and Simenhoff, 1965). Nitrosamines in concentrations of 2 to 3 $\mu\text{g/liter}$ have been found in the urine of patients with Proteus mirabilis and Escherichia coli infections (Brooks et al., 1972; Radomski et al., 1978). Unfortunately, there were no adequate controls for possible artifactual formation of NDMA during analytical chemistry procedures. In the study by Brooks et al., urine was acidified to pH 2 and then extracted with chloroform. If amines and nitrite were present in the urine, NDMA would have been formed at this stage. In the study by Radomski et al., the urine was frozen prior to analysis without the inclusion of an artifact inhibitor.

Although several other workers have reported the presence of nitrosamines in the normal urine of healthy volunteers (El-Merzabani et al., 1979; Hicks et al., 1977; Kakizoe et al., 1979), they may have underestimated the analytical problems encountered when determining volatile nitrosamines in urine at the 0.02 to 2 $\mu\text{g/liter}$ level. Eisenbrand et al. (1981) detected nitrosamines in urine of a volunteer who had consumed a liter of beer containing 60 μg of NDMA; however, they could not detect nitrosamines at 0.1 $\mu\text{g/}$

1980; Lakritz et al., 1980). All three groups of investigators occasionally reported the presence of nitrosamines in the blood prior to consumption of a meal. Fine et al. and Kowalski et al. demonstrated that the level of nitrosamines increased immediately following ingestion of a meal. Lakritz et al. (1980) did not make similar measurements. As with urine and feces, it is extraordinarily difficult to be certain that no artifacts have been formed in blood. None of these three studies included the critical artifact test of assessing the amount of a marker amine that is converted to a nitrosamine. Eisenbrand et al. (1981) have not been able to reproduce these data when they incorporated appropriate controls. Thus, the initial finding of nitrosamines in blood by Fine and his colleagues could have resulted from the ingestion of preformed nitrosamines presumed to be present in the beer consumed with the meal rather than from endogenous formation (Eisenbrand et al., 1981).

Recently, Ohshima and Bartsch (1981) have developed a technique for estimating the extent of in vivo nitrosamine formation in humans who have ingested proline with nitrate by assaying urine samples, collected for 24 hours following ingestion, for nitroso-proline (NPRO), a nonvolatile nitrosamine. They believe that their approach is sensitive and practical for two reasons: NPRO is not carcinogenic in animals and it is not readily metabolized (80% is excreted in the urine within 24 hours). They reported a background NPRO level of less than 3 $\mu\text{g/liter}$ of urine (3 $\mu\text{g/person/day}$), which was not significantly increased by ingesting proline alone or nitrate alone. However, ingestion of red beet juice (containing up to 325 mg of nitrate) followed 30 minutes later by proline (500 mg) produced readily detectable levels of NPRO (0.016 to 0.030 mg).

The data reported by Ohshima and Bartsch compare well with the estimates of in vivo formation of NPRO derived by Fine et al. (1981) from the kinetics data on the rate at which NPRO is formed from proline and nitrite in vitro (Mirvish et al., 1973). At the low rates of conversion observed by Ohshima and Bartsch (0.002% of the nitrate and 0.004% of the proline), the rate of reaction can be assumed to be linear over the time of the reaction. The amount of NPRO formed during the time interval Δt can be determined by the following equation:

$$\Delta [\text{NPRO}] = \Delta t \, k_2 [\text{amine}] [\text{nitrite}]^2 \quad (1)$$

1975), the equation can be summarized as:

$$\sum_{1-5 \text{ hr}} (\text{NPRO, in } \mu\text{g}) = \sum_{1-5 \text{ hr}} 0.04865 (\text{nitrite, in mg})^2 \quad (2)$$

The data of Ohsima and Bartsch are compared in Figure 8-4 with the amount of NPRO calculated by these workers. The center line represents results of calculations based on 1.0 mg of nitrite produced in 50 ml of saliva each hour per 100 mg of nitrate ingested (Spiegelhalter et al., 1976). Reasonable lower and upper bounds for salivary nitrite levels, considering person-to-person and day-to-day

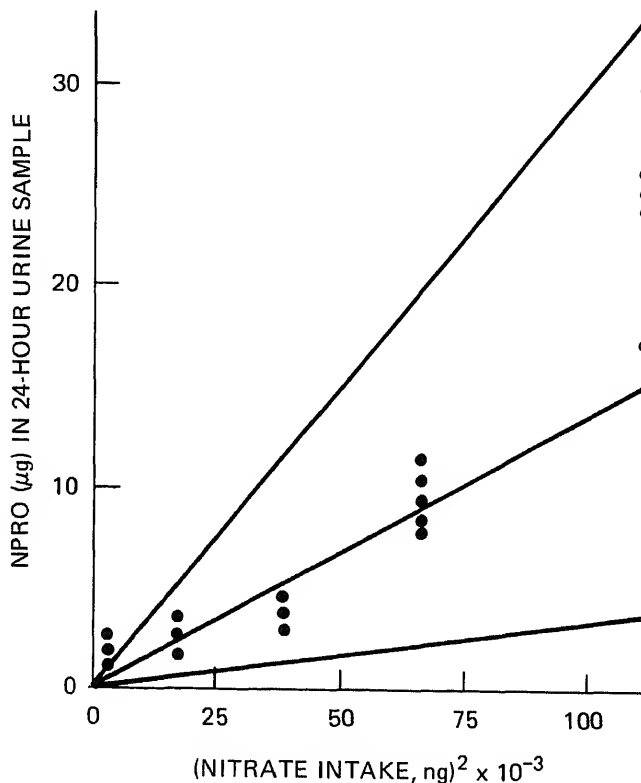


FIGURE 8-4. Plot of NPRO produced by endogenous synthesis versus the square of the nitrate concentration in the diet. The data points (●) are from Ohshima and Bartsch (1981). The straight lines were calculated using equation (2) for three levels of conversion of ingested nitrate to salivary nitrite. The top line represents 1.5 mg of salivary

mental results of Ohshima and Bartsch within the limits of experimental error, thereby validating the effectiveness of the model to predict the extent of endogenously formed NPRO. Additional confidence in the model is provided by Tannenbaum (personal communication, 1981), who used the approach of Ohshima and Bartsch and obtained essentially similar results.

These findings and their remarkable correlation with in vitro kinetics have several important implications. First, it seems likely that both volatile and nonvolatile nitrosamines are formed in humans. Second, the extent of this in vivo formation, at least in the stomach, can be readily calculated from the nitrosation kinetics of the amine at acid pH. Third, the calculations can be applied to both volatile and nonvolatile nitrosamines. Fourth, the experimental findings of the percentage of nitrate converted to nitrite can be used to estimate the body burden of nitrite from ingested nitrate.

Table 8-3 shows the amounts of endogenously produced NPRO calculated by using the data for nitrate and nitrite in Tables 5-20 and 5-21 and assuming that the daily amine intake is 4,000 mg. (Actual intake of nitrosatable amines is unknown and could, of course, be significantly different from this estimate.) For the purposes of calculation, intake of nitrate, nitrite, and amino compounds is assumed to be equal at major meal times, i.e., 2,000 mg of proline per meal twice daily. It is also assumed that dietary amines are nitrosated at the same rate as proline. This is an oversimplification since Mirvish (1975) showed that the rate at which proline is nitrosated under acidic conditions lies between that of dimethylamine (which is 22 times slower than proline) and morpholine (which is 11 times faster than proline). The rates for amines such as aminopyrine and piperazine are 2,000 times faster than that of proline. Another assumption, which may or may not be valid, is that the stomach is the primary site for nitrosation reactions in vivo.

Despite the inexact nature of the calculations presented, the committee believes that they do permit the comparison of relative exposures that stem from different lifestyles. Five different lifestyles are considered: (1) the average intake for the general population; (2) a high-cured-meat diet, assuming 4 times the average intake of cured meats; (3) a vegetarian diet with 4 times the normal vegetable intake, excluding meat; (4) average intake including 160 mg nitrate/person/day from nitrate-contaminated drinking water; and (5) a high-cured-meat, high-nitrate-water diet. The five categories are arbitrary and are not meant to be accurate estimates. They were selected to illustrate the impact of lifestyle on endogenous synthesis of nitrosamines, and they can be modified to include other lifestyle factors to develop an estimate for other populations.

Amount of Nitrosamines Produced In Vivo from Proline and
Various Levels of Nitrate and Nitrite

Compound	Intake, mg/Person/Day, by Type of Diet				
	Average	High Cured Meat ^a	Vegetarian ^b	Nitrate-Rich Water ^c	High Cured Meat ^a and High-Nitrate Water ^c
Exogenous nitrate ^{a,b,c}	75	78	268	233	236
Exogenous nitrite ^{a,b,c}	0.77	1.7	0.77	0.77	1.7
Nitrosoproline Produced Endogenously, μg					
Endogenous NPRO, with ascorbic acid ^{d,e}	0.32	0.63	2.4	10	12
Endogenous NPRO, without ascorbic acid ^{d,f}	2.2	3.13	22	17	20

^aAssuming 4 times the normal amount of cured meats ingested. From Tables 5-20 and 5-21.

^bAssuming 4 times the normal amount of vegetables ingested and no meat. From Tables 5-20 and 5-21.

^cFrom Table 5-20. Nitrate-rich water estimated as 100 mg/liter and assuming daily intake to be 1.6 liters or 160 mg of nitrate.

^dAssumes one-half of the vegetables and all fruit eaten raw. Thus, coingested ascorbic acid combines with half of the nitrite produced from 75 mg nitrate, except for the vegetarian diet, where it would react with half of the nitrate from 268 mg of nitrate.

^eAssumes 2,000 mg of proline ingested per meal twice a day and that half of the daily nitrate and nitrite are ingested at each meal. The rate is calculated from in vitro kinetics in a stomach with a 900 ml capacity. Equation reduces to $\mu\text{g NPRO} = 0.04865 (\text{mg NO}_2^-)^2$.

^fEndogenous NPRO produced, calculated by equation in footnote e, assuming that no ascorbic acid is available to react with nitrite.

The lower NPRO levels shown in the table were calculated by assuming that there was enough ascorbic acid in the vegetables and fruit to react with half the nitrite in the stomach. The higher NPRO values were calculated by assuming that all the nitrite present in the stomach was free to react, without competition from ascorbic acid. A situation like this could occur when meats are overcooked. These calculated daily NPRO exposures indicate that the ratio of ascorbic acid to nitrate in vegetables is of critical importance. Shimizu and Bartsch observed complete inhibition of endogenous NPRO synthesis when the ascorbic acid/nitrate molar ratio exceeded 1.0. Although the molar ratio is less than 0.1 for vegetables such as lettuce, beets, and celery, it exceeds 3.0 for vegetables such as brussels sprouts and peas. Thus, large

of amines is 4,000 mg per day; second, that all dietary amines behave like proline; and third, that endogenous nitrosation is restricted to the stomach. A further limitation to this comparison is that, apart from tobacco products, the data base for exogenous exposures is limited to volatile nitrosamines, whereas estimates of endogenous exposures include both volatile and nonvolatile nitrosamines.

The estimates in Table 8-4 imply that the endogenous synthesis of nitrosamines for the average nonsmoker contributes from 15% (reasonable ascorbic acid intake in vegetables and fruit) to 58% (no ascorbic acid whatsoever in the diet) of total nitrosamine exposure. This finding is substantially different from the earlier estimates that endogenous synthesis greatly exceeds exogenous exposures (Tannenbaum, 1980). The greatest exogenous exposures to nitrosamines would occur in workers in certain industries, namely rubber factories and leather tanneries, and in smokers. The greatest endogenous exposures would occur in individuals consuming high-nitrate water and large quantities of cured meats.

Bacteria-Mediated Catalysis of Nitrosation Reactions. In vivo formation of N-nitroso compounds can be increased or decreased by a number of agents. For example, under certain conditions, some phenols may catalyze the nitrosation reaction, whereas ascorbic acid and α -tocopherol may inhibit it. Studies of the mechanisms and environmental distribution of the modifiers of nitrosation reactions are discussed in Chapters 4 and 6, respectively. Other studies indicate that bacteria may also catalyze the in vivo formation of N-nitroso compounds and epidemiological studies have suggested that bacterial catalysis of nitrosation reactions may be involved in the induction of stomach and bladder cancers in humans.

Three years after Sander (1968) demonstrated that bacteria could catalyze the nitrosation reaction, Hawksworth and Hill (1971) reported that five strains of E. coli formed nitrosamines when incubated aerobically with the secondary amines diphenylamine, dimethylamine, diethylamine, piperidine, pyrrolidine, and N-methylaniline. They also demonstrated that nitrosamines were formed only in the presence of bacteria and that the reaction was not due to acid catalysis since formation of nitrosamines occurred at pH 6.5. Subsequently, however, other investigators have demonstrated that bacterial enhancement of nitrosation is greater at high pH than at low pH (Collins-Thompson et al., 1972; Drasar and Hill, 1974; Kunisaki and Hayashi, 1979). This results from bacterial production of acid, which then permits an increase in the rate of nonenzymatic nitrosation in the culture medium.

Exposure of Humans to N-Nitroso Compounds from Exogenous
and Endogenous Sources^a

<u>Source</u>	<u>Exposure, μg/Person/Day</u>	
	<u>Average</u>	<u>High</u>
<u>Exogenous Exposure:</u> ^b		
Cosmetics (volatile)	0.41	0.82 ^c
Car interiors (volatile)	0.20	0.50
Dietary (volatile)	1.1 ^d	
Beer	0.97	3.9 ^e
Bacon	0.17	0.68 ^f
Tobacco smoke (volatile and nonvolatile)	~ 17	~ 35 ^g
<u>Occupational Exposure:</u> ^h		
	<u>Range</u>	
Leather tanning (volatile)	20 - 180	440
Rubber factory (volatile)	50 - 130	250
Rocket fuel factory (volatile)	10 - 50	260
<u>Endogenous Exposure (Dietary):</u> ⁱ		<u>Average</u>
Average (U.S.) (volatile and nonvolatile)	0.32 - 2.2	1.3
High cured meat (volatile and nonvolatile)	0.63 - 3.3	2.0
Vegetarian (volatile and nonvolatile)	2.4 - 22	12
High-nitrate water (volatile and nonvolatile)	10 - 17	14
High cured meat and high- nitrate water (volatile and nonvolatile)	12 - 20	16

^aEstimates for exogenous exposure based mainly on data for volatile nitrosamines (apart from tobacco smoke). The extent of exposure to exogenous nonvolatile nitrosamines is still unknown.

^bTaken from Table 7-17.

^cAssumes twice the amount of cosmetics used.

^dTaken from Table 7-15.

^eAssumes 4 times the amount of beer consumed.

^fAssumes 4 times the amount of bacon consumed.

chylamine and sodium nitrite at neutral pH. Subsequently, es et al. (1972) observed that the formation of nitrosamine decreased with nitrite concentration, but that it was inhibited if the intestinal contents were autoclaved or boiled or when the broad-spectrum antibiotic neomycin was added to the incubation. Bacteroides and Clostridia would not be affected by neomycin presumably, are not involved in catalyzing nitrosation. The addition of riboflavin increased the formation of nitrosamines by approximately 30% by stimulating the enzyme systems of the anaerobic bacteria. The authors also pointed out that the mixed fecal cultures used in their studies produced higher levels of nitrosamines than did the pure cultures used by Hawksworth and Hill (1971) under aerobic conditions.

Other examples of bacteria-mediated nitrosation have been reported by Archer et al. (1978), who found that the rates at which certain dialkyl nitrosamines were formed were accelerated at 1.5 in the presence of various microorganisms such as gram-positive and gram-negative bacteria as well as yeast. However, in these studies, rate enhancements were similar for both boiled and intact cells, suggesting that there was a hydrophobic interaction of precursor amine with a cellular constituent, such as a component of the cell wall. In support of this idea, the authors found that the particulate fraction of sonicated cells was responsible for 80% of the catalysis. Further evidence that a hydrophobic interaction was responsible came from structure-activity studies where rate enhancement increased with increasing alkyl chain length of the amine (Archer et al., 1978).

Hill et al. (1973) summarized clinical situations in which bacteria-catalyzed nitrosamine formation was implicated in disease causation. Likely sites of such catalysis include the infected urinary bladder and the achlorhydric stomach (Hill, 1979). For example, evidence that bacteria-mediated nitrosamine formation occurs in the infected bladder has been provided by studies in rats (Hawksworth and Hill, 1974). These authors also found that labeled NDMA injected into the bladder was not totally excreted in the urine but was absorbed into the circulating blood. It was found primarily in the liver, kidney, lung, stomach, and small intestine. In another study, Fong et al. (1980) studied infected bladder infections in rats with E. coli and exposed the infected rats to nitrate and aminopyrene. Mutagens requiring metabolic activation were detected in these animals, but not in non-infection-free control animals given nitrate and aminopyrene. There was also an apparent increase in the number of tumors in infected animals.

in the more frequent formation of carcinogens. Epidemiological studies of bladder cancer in humans are discussed in detail in Chapter 9.

Gastric achlorhydria creates conditions that favor a profuse gastric flora because of the elevated pH (Tannenbaum *et al.*, 1977). Thus, individuals with that condition may be at high risk of gastric cancer since the bacteria could catalyze the formation of N-nitroso compounds from ingested secondary amines/amides and nitrate/nitrite (Hill *et al.*, 1973). In addition, bacteria may aid in the nitrosation of drugs in the saliva (Spiegelhalder *et al.*, 1976). Mirvish (1975), Lijinsky and Epstein (1970), and Rao (1980) provided lists of nitrosatable drugs currently in use (Chapter 6). Some of the nitrosamines resulting from nitrosation of those drugs are carcinogenic in animals.

Bacteria-mediated catalysis of nitrosation reactions can also occur exogenously. Ayanaba and Alexander (1973) described studies in which microorganisms isolated from soil or sewage formed NDMA during incubations with trimethylamine and nitrate at neutral pH. These results suggest that the presence of nitrosamines or their precursors in sewage or soil may be a potential environmental hazard to humans.

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

In persons with normal gastric acidity, nitrate is converted to nitrite mainly in saliva by the action of oral microflora. Ingested nitrate is absorbed from the gastrointestinal tract and is transported via the plasma to the salivary glands. Nitrate, thiocyanate, and iodide appear to share a common active transport system in the salivary glands of animals and, thus, compete for uptake from the circulatory system. Nitrate secreted by the salivary glands is approximately 25% of the nitrate ingested and approximately 20% of salivary nitrate is reduced to nitrite. Thus, 5% of ingested nitrate is reduced to nitrite in the saliva. Using this conversion factor, the committee estimated that approximately 72% of the nitrite load to which the upper gastrointestinal tract is exposed is derived from ingested vegetables, and about 9% from cured meats. When gastric pH approaches neutrality, the presence of microorganisms could also permit extensive reduction of nitrate in the stomach, resulting in a greatly increased gastric nitrite load.

Although the active transport of nitrate from the circulatory

but it has been found in the urine of patients with urinary tract infections.

Nitrate balance studies in humans have indicated that the quantity of nitrate excreted in the urine can exceed the amount ingested when these amounts are small. Heterotrophic nitrification of ammonia or organic nitrogen has been postulated as a mechanism to account for this difference; however, subsequent studies with labeled nitrate in rodents indicate that such microbial reactions are not likely. Moreover, the analytical method used might have led to an underestimate of the nitrate content of ingested food, water, and air. Recent studies suggest that mammalian synthesis of nitrate partially explains the excess nitrate excreted in the urine.

The organotropic, toxic, and carcinogenic effects of nitrosamines probably result from preferential metabolism by specific organs. However, although tumor induction occurs only in organs in which alkylation takes place, the nature of the alkylating molecule is not known for a single nitrosamine. The primary reaction required for activation may involve an enzyme-mediated α -hydroxylation. Studies of the mechanisms of activation also suggest that oxidative dealkylation is necessary for the toxic and carcinogenic action of nitrosamines and that cytochrome P450 metabolizing enzymes are involved.

Efforts to explain the carcinogenicity of various nitrosamines have focused on alkylation of DNA, and some 11 different nucleoside and alkylphosphotriester adducts have been found. However, the balance among adduct formation, repair mechanisms, and cellular replication may be the most important determinant of the carcinogenic process. Conditions that accelerate replication or delay repair could enhance miscoding, thereby increasing the number of mutations and enhancing the carcinogenic effect, whereas conditions that delay replication or enhance repair efficiency would have the reverse effect. When considering the importance of repair mechanisms, one must take into account the fact that humans repair damaged DNA more efficiently than do other species and that repair is more efficient at low doses of carcinogen.

In vivo formation of N-nitroso compounds has been studied extensively in laboratory animals. Sodium nitrite and an amine coadministered to animals have led to the formation of nitrosamines, which produce toxic effects (tumors or acute damage to target organs). In humans, the evidence is sparse. Nitrosamines have been measured in a variety of biological samples (e.g., feces, urine, and blood);

of human subjects following ingestion of proline and nitrate. This suggests that in vivo synthesis of these compounds does occur in humans. Based on these preliminary findings and data on average ingestion of nitrate and nitrite from Chapter 5, the committee has calculated rough estimates of the amounts of N-nitroso compounds formed in vivo based on the limited data available on exposure to nitrate and nitrite and endogenous formation of N-nitroso compounds in humans. For the average population, the amount of preformed nitrosamines in the diet is roughly equivalent to the amount formed in vivo from the intake of nitrate and nitrite. However, for special population groups, such as those ingesting high-nitrate water, the increased intake of nitrate could lead to a corresponding increase in the amount of nitrosamines formed in vivo.

Bacteria-mediated formation of nitrosamines may be important in certain target tissues, such as the achlorhydric stomach and the infected bladder, which are colonized by bacteria. Although the precise role of bacteria in nitrosation reactions is not known, it has been suggested that a hydrophobic interaction of a precursor amine with a bacterial cellular constituent (e.g., cell wall) may catalyze the reaction if the amine is lipophilic.

Although much is known about the metabolism of nitrate, nitrite, and N-nitroso compounds, many remaining uncertainties will have an important impact on the overall conclusions on the possible health effects of exogenous exposures to these compounds in humans -- a topic that will be considered in detail in the following chapter. These uncertainties include the extent and importance of the endogenous synthesis of nitrate, nitrite, and N-nitroso compounds, as compared to exogenous exposure. In addition, more detailed information is needed on the metabolism of N-nitroso compounds, including metabolic activation, deactivation, and cellular repair mechanisms for genotoxic effects in the human. Thus, although the data presented in this chapter may indicate the possible significance of the endogenous synthesis of N-nitroso compounds following exposure to exogenous nitrate/nitrite, many uncertainties remain so that the overall significance to human health of the experimental findings is still unclear.

Based on the data presented in this chapter, the committee recommends the following:

1. Additional studies should be conducted to increase understanding of the metabolism of nitrate in humans and to clarify the role of mammalian synthesis of nitrate and nitrite. Also requiring clarification is the role of bacteria in the reduction of nitrate to nitrite and in the formation of N-nitroso compounds.

the nitrosation-inhibiting effects of normal dietary constituents should be studied further to determine the extent of their effects in vivo. Specifically, further research is needed to determine the amount of nitrite that is destroyed in the human stomach and the extent to which nitrosation reactions are modified by the various inhibitors. Attention should also be directed toward in vivo interactions among inhibitors, catalysts, and other food-derived substances.

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ADVERSE EFFECTS OF NITRATE, NITRITE, AND N-NITROSO COMPOUNDS

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ADVERSE EFFECTS OF NITRATE, NITRITE, AND N-NITROSO COMPOUNDS

Exposure to nitrate, nitrite, and N-nitroso compounds has been implicated in the causation of a variety of diseases in humans and in other species. The first section of this chapter examines studies concerning the role of these compounds in the causation of cancer. This is followed by a discussion of the data on their mutagenicity in bacterial and mammalian systems. The third section reviews reports of other toxic effects following exposure to these agents. After weighing the evidence pertaining to the adverse effects of these compounds, the committee developed specific recommendations, which appear at the end of this chapter.

CARCINOGENICITY

An evaluation of the data from studies of human populations is followed by a review of studies on laboratory animals. Where possible, deficiencies in the data are noted in the discussion.

Studies of Humans and Related Evidence from Laboratory Studies

Research on the role of nitrate, nitrite, and/or N-nitroso compounds in the induction of cancer in humans has focused mainly on hypotheses that cancers of the stomach, esophagus, and nasopharynx are attributable to exposure to these compounds. To provide some perspective on the limited data obtained from epidemiological research, discussions of studies in humans are augmented by descriptions of results obtained in pertinent laboratory studies.

Stomach Cancer

Some investigators have hypothesized that stomach cancer can be attributed to exposure to N-nitroso compounds (see Chapter 8), especially nitrosamides (e.g., nitrosoureas), which are formed endogenously by the reaction of nitrosatable substrates and nitrite (Correa et al., 1975a; Haenszel and Correa, 1975; Mirvish, 1971, 1977; Weisburger, 1979; Weisburger and Raineri, 1975). The committee believes that this hypothesis is plausible for four reasons:

• The feeding of nitrosoureas and nitrosocarbamates to rodents has produced a low incidence of gastric adenocarcinoma, which resembles stomach cancer in humans (Druckrey and Landschütz, 1971; Druckrey et al., 1968, 1970, 1971; Maekawa et al., 1976; Ogiu et al., 1975). Moreover, a related compound, 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), is the strongest known carcinogen for stomach cancer in rodents and dogs (Sugimura and Kawachi, 1973, 1978).

Nitrate is converted to nitrite in the mouth and the achlorhydric stomach (see Chapter 8); hence, it could lead to the formation of N-nitroso compounds in the stomach. Laboratory studies have provided support for this mechanism of stomach cancer induction (Franklin and Skoryna, 1971; Ishiwata et al., 1975) and can be compared to attempts to correlate the incidence of stomach cancer with exposure to high levels of nitrate in humans.

Modan et al. (1974) reported a correlation between the consumption of high-starch, low-protein diets and a high incidence of stomach cancer in humans. These observations correspond to the findings of Mirvish et al. (1980b), who studied the formation of nitrosomethylurea in the stomach of rats fed diets containing methylurea and nitrite. When a low-protein diet was fed to these animals, the concentration of nitrosomethylurea in the stomach was almost 4 times greater than when a high-protein diet was used. The investigators suggested that the protein buffered the stomach contents, thereby causing the observed increase in pH and, hence, inhibiting the nitrosation.

The importance of an elevated gastric pH in nitrosation reactions is not yet clear because nitrosation may be enhanced by two mechanisms—one at low pH and the other at high pH. For example, low gastric pH would favor the production of nitrosamides because chemical nitrosation of amides increases tenfold for each unit drop in pH (Mirvish, 1975; Mirvish et al., 1980b). On the other hand, a high gastric pH (achlorhydria) could also favor the production of N-nitroso compounds because this condition allows the growth of bacteria that reduce nitrate to nitrite, thereby providing an increased level of nitrosating species (Cuello et al., 1976; Ruddell et al., 1976).

Perhaps both views are valid but apply at different stages of gastric carcinogenesis. Thus, an initially low pH may favor the production of nitrosamides, which cause the development of precancerous

lesions. Nitrosation may then continue if the nitrite level is sufficiently high to counterbalance the inhibitory effect of high pH on nitrosation. The resultant nitrosamides may cause the precancerous lesions to develop into cancer. Alternatively, nitrosation might occur when the nitrite is transferred from a part of the stomach with a high pH to one with a low pH; or nitrite might be formed at a high pH at one time of the day, and this could cause nitrosation later when acid is secreted.

Studies with the drug cimetidine may eventually shed some light on the role of pH in nitrosation reactions. Cimetidine is a cyano-guanidine compound that can be nitrosated to give mutagenic N-nitroso compounds, which are similar in structure to MNNG (Bavin et al., 1980; Foster et al., 1980; Ichinotsubo et al., 1981). It is used to reduce gastric acidity in the treatment of peptic ulcers. Apparently, nitrite accumulates in the stomachs of ulcer patients treated with cimetidine (Ruddell, 1981).

The importance of the accumulation of nitrite in the stomach has been investigated. Stemmerman et al. (1980) detected direct-acting mutagens in mucosa removed from the human stomach during surgery. The mutagens were later identified as the N-nitroso derivatives of three drugs (hydroxyzine hydrochloride, diazepam, and cimetidine) that had been administered to the patients (Rice et al., 1981; Stemmerman et al., 1981). Methylation of DNA by nitrosocimetidine has been observed in vivo and in vitro (Gombar et al., 1981; Jensen and Magee, 1980). Additional studies are being conducted to determine whether nitrite can produce large amounts of nitrosocimetidine in the gastric contents (despite the relatively high pH) and whether nitrosocimetidine is carcinogenic in the stomach (Elder et al., 1979; Guslandi, 1979; Hawker et al., 1980; Mullen, 1979; Reed et al., 1979; Ruddell, 1981; Taylor et al., 1979). In one preliminary report, nitrosocimetidine did not cause tumors in laboratory animals (Preussmann, personal communication).

Table 9-1 lists the age-adjusted death rate per 100,000 population for stomach cancer in males and in females and various estimates of ingested nitrate and nitrite ions for the same populations (see footnotes b-e for exceptions). To obtain these ingestion data, investigators used a variety of methods, ranging from assays of prepared meals to estimates based on food consumption tables plus published estimates of the nitrate and nitrite content of foods. Moreover, the assays have been performed as much as a decade apart and by different methodologies in different countries. This table also does not consider the lag period in the induction of cancer and

TABLE 9-1

Relationship between Mortality from Stomach Cancer and Ingestion Nitrate and Nitrite in 11 Countries

Country	Stomach Cancer (Age-Adjusted Death Rate per 100,000 Population) ^a		Estimates of Consumption Ranges and (Averages), mg/Person/Day		References
	Male	Female	Nitrate	Nitrite	
Japan	56.6	29.0	(297) (385) 380 - 490 44 - 864 (218) (388) ^c (271) ^c	(1.5) 0.7 - 10 ----- ----- <0.1 - 1.3 ----- -----	Kawabata <i>et al.</i> , 1979 Harada <i>et al.</i> , 1975 Ishidate, 1977, cited in Endo <i>et al.</i> Ishiwata <i>et al.</i> , 1978 Maruyama <i>et al.</i> , 1979 Maruyama <i>et al.</i> , 1979
Czechoslovakia	33.3	16.6	(142)	----	Turek <i>et al.</i> , 1980
Federal Republic of Germany	27.1	14.1	(75) ^d (49) ^e	(3.3) (1.7) ^e	Selenka and Brand-Grimm, 1976 Selenka and Brand-Grimm, 1976
Yugoslavia	24.0	11.5	(156) ^f	(6.5) ^f	Adamovic, 1979
Netherlands	21.4	9.7	(110)	(2.8)	Stephany and Schuller, 1980
United Kingdom	19.7	9.0	(58) ^g (115) ^h	----- -----	Ashton, 1970 Walker, 1975
Switzerland	18.1	10.2	(91)	----	Tremp, 1980
Norway	17.4	9.8	(32) ⁱ (48)	(1.1) ^h (0.11)	Gislason and Dahle, 1980 Høyem, 1974
Sweden	16.3	8.3	(150) 24 - 68 (42) 110 - 190	0.5 - 5 0.9 - 7.3 (3.7) 2 - 10	Sandberg, 1978, cited in Slorach, 1978 Jägerstad and Nilsson, 1976, Jägerstad <i>et al.</i> , 1976 Boström and Tammelin, 1981
Denmark	14.8	8.1	(54)	(0.74)	Statens Lægemiddelinstitut
United States	7.2	3.7	(75)	(0.78)	Tables 5-20 and 5-21

^aFrom American Cancer Society, 1980.^bResidents of Tokyo.^cResidents of Nagano Prefecture.^dIn an area with nitrate-free drinking water.^eCalculated from food consumption statistics.^fResidents of Serbia.^gIncludes only meat products, water, and vegetables (excluding potatoes).^hExcludes contributions from cereals, fruits, and dairy produce, and assumes maximal estimates for nitrate contents of meat products and water.ⁱIncomplete tabulation; vegetables are listed as the only significant source of nitrate and cured meats and potatoes as the sources of nitrite.

consumption of salted dried fish products conducted in various countries throughout the world.

Japan. Japan has the highest reported incidence of stomach cancer in the world. This high rate has been associated with the consumption of salted dried fish products known to contain high levels of certain secondary amines (Singer and Lijinsky, 1976) and other precursors of N-nitroso compounds (see below). Salt has been suggested as a promoting factor for this disease (Haenszel and Correa, 1975; Joosens and Geboers, 1981; Sato et al., 1959) and has been demonstrated to be a cocarcinogen in test animals (Capoferro and Torgersen, 1974; Kinoshita, 1969; Tatematsu et al., 1976). Japanese who immigrate to Hawaii continue to be at high risk for stomach cancer, but the risk is substantially lower for the next generation (Nisei) (Haenszel et al., 1972).

Epidemiological studies of these immigrants indicate an association between a high incidence of stomach cancer and the consumption of salted dried fish and salt-pickled vegetables (Haenszel et al., 1973). In Japan, this association was not confirmed, but investigators reported an elevated risk for stomach cancer among Japanese farmers who consume both types of food more frequently than do other population groups (Haenszel et al., 1976b). Other important sources of N-nitroso compound precursors were not ruled out. For example, Japanese farmers, more often than other occupational groups in that country, drink well water that could contain elevated levels of nitrate. They also consume fewer vitamin-C-containing fruits and vegetables. Vitamin C intake has been hypothesized to be inversely correlated with the incidence of gastric cancer because of its inhibitory effect on nitrosation reactions (Chapters 4 and 8). Kolonel et al. (1979) have also reported a negative association between fresh fruit consumption and gastric cancer in a prospective study conducted in Hawaii, which included Japanese immigrants.

In contrast to the studies linking stomach cancer to exposure to nitrate or nitrite, there is no evidence linking large bowel cancer to such exposures (Haenszel et al., 1973). Table 9-2 summarizes the major results and conclusions of epidemiological studies of gastrointestinal cancer among the Japanese.

Laboratory studies lend some support to the suggestion that salted dried fish plays a role in the etiology of gastric cancer. For example, treatment of a Japanese dried bonito fish product with excess nitrite and then with acid ("nitrosation-dinitrosation") produced 25 mg of methylurea per kilogram. The carcinogen nitrosomethylurea was formed after treatment with excess nitrite alone (Mirvish

TABLE 9-2

Gastrointestinal Cancer Among Japanese

Methods	Results and Interpretation	References
Interviews of 220 Japanese stomach cancer patients and 440 matched controls	Immigrants (Issei) continued to be at high risk, but risks declined in their children (Nisei). Association with pickled vegetables and dried salted fish; inverse association with vegetables, especially tomatoes, celery, and corn. Dried fish said to contain high concentrations of secondary amines.	Haenszel et al., 1973
Pathology review of 407 cases from Japan and 256 from Hawaii	Incidence of diffuse carcinoma was similar; incidence of intestinal, mixed, and other types was lower in Japan.	Correa et al., 1973
Interviews of 179 Japanese colorectal cancer patients and 357 matched hospital controls	Unlike stomach cancer, results were similar for immigrants and their children. Bowel cancer patients ate meat, legumes, and starches more frequently. Relative risk was as high or higher for meats containing little or no nitrate, e.g., preserved pork products, and there was no association with dried and salted fish rich in nitrate and nitrite.	Haenszel et al., 1973
Interviews of 783 stomach cancer patients and	Elevated risk among farmers. Hawaiian Japanese finding of increased salted dried fish and salt-pickled vegetable	Haenszel et al., 1976b

product treated with excess nitrite at pH 3 was found to contain a direct-acting mutagen, which was not nitrosomethylurea (Marquardt et al., 1977a; Mower and Weisburger, 1978). The mutagen has not yet been identified. When the same product was fed to 12 rats, it produced glandular stomach tumors in five of the animals (Weisburger et al., 1980).

Mutagens are also produced in similarly treated beans and beets, which are eaten in Colombia and Chile. Both of these countries also have high rates of gastric cancer (see below) (Marquardt et al., 1977a,b).

Colombia. Colombia has one of the highest rates of mortality from stomach cancer, especially among persons living at high altitudes in certain rural areas (Correa et al., 1975a). Cuello et al. (1976) reported a fourfold geographic variation in the incidence of gastric cancer within this country. Correa et al. (1970, 1975a, 1976) have shown geographic correlations of stomach cancer incidence not only with nitrate content of well water but also with the prevalence of atrophic gastritis and, to some extent, with the nitrate in urine and saliva. In carefully conducted studies involving gastroscopy of volunteers, they found three-fourths of the high-risk population to have superficial gastritis or other dysplasias. In contrast, a much lower frequency was found in persons from the lower risk areas of Colombia.

Correa and his colleagues have suggested that superficial gastritis, atrophic gastritis, and intestinal metaplasia are precursor lesions for gastric cancer. They described in detail the histopathology of the gastric lesions, beginning with the normal mucosa and progressing through various degrees of dysplasia to stomach cancer. Analyses of gastric fluid of individuals with atrophic gastritis indicated that nitrite levels were elevated in patients whose gastric pH was above 5 (Correa et al., 1979). Since nitrosation reactions could occur more readily at higher nitrite concentrations, the formation of N-nitroso compounds may be a key factor in the development of gastric cancer. Data from case-control studies of persons with dysplasia indicate that a history of a high corn diet is associated with dysplasia but ingestion of lettuce, which contains ascorbic acid (vitamin C), is inversely associated. Additional data supporting the role of N-nitroso compounds in gastric cancer were reported by Montes et al. (1979), who detected a direct-acting mutagen, possibly a nitrosamide, in the gastric juice of fasting subjects from an area with a high incidence of gastric cancer, and by Hawksworth et al. (1975), who observed concentrations of nitrate as high as 146 mg/liter in water supplies

Table 9-3 summarizes the major results and conclusions of epidemiological studies of gastric cancer conducted in Colombia.

Chile. Chile has natural deposits of nitrate, and for decades has used large amounts of these compounds in fertilizers. Gastric cancer mortality in 1960, 1962, and 1964 was statistically correlated with the use of nitrate fertilizers from 1960 to 1964 for each province (Zaldívar, 1977; Zaldívar and Robinson, 1973; Zaldívar and Wetterstrand, 1975). An association was found between mortality from stomach cancer and the proportion of farmers using nitrate-containing fertilizers in each province; in contrast, there was no association with miners. This association remained significant when data from the industrialized provinces were excluded in order to eliminate the interference of occupational or industrial factors.

A more detailed study was conducted by Armijo and Coulson from 1957 to 1972. These investigators found no association between stomach cancer and exposure to nitrate in drinking water, but they noted that the levels of nitrate throughout Chile were well below 100 mg/liter -- the 1972 maximum acceptable level established by the World Health Organization (Armijo and Coulson, 1975). However, they did report a high correlation between the gastric cancer mortality in each province and the estimated per capita exposure to nitrate from fertilizers.

Armijo et al. (1981b) interviewed 389 gastric cancer patients at the gastroenterology clinics of seven Santiago hospitals and 845 controls. They found that the patients had resided in high risk areas during their early lives for longer periods than had controls. Gastric cancer was also found to be associated with previous occupation in agriculture.

Table 9-4 summarizes the major results and conclusions of epidemiological studies of gastric cancer in Chile. The committee, however, has some reservations about these data, particularly that inadequate attention was directed toward the latent period in cancer induction and, in one study (Cuello et al., 1976), the validity of the sampling methods used to measure urinary nitrate is doubtful.

England. In an epidemiological study conducted in England, Hill et al. (1973) correlated differences in stomach cancer mortality rates with the nitrate content of drinking water in two towns. They found that the town with the higher level of nitrate (average, 10 mg/liter) had the higher mortality from this cancer. The ratios of observed to expected gastric cancer cases were 1.3 for males and 1.7 for females. Hill and coworkers, however, did not consider

TABLE 9-3

Gastric Cancer in Colombia

Methods	Results and Interpretation	References
Study of 322 cases from Colombia and Mexico City.	Two histological types: "intestinal" (glandular) and "diffuse" (undifferentiated). Intestinal more common in high risk areas (52% vs 36% in lower risk areas) and may follow precancerous lesions.	Munõz et al., 1968
Cali registry incidence rates were correlated with intestinal metaplasia in natives vs immigrants.	Intestinal metaplasia correlated with gastric cancer incidence. Authors hypothesized that premalignant change in young predispose to later cancer.	Correa et al., 1970
Hypothesis based on other studies.	Hypothesis that gastric atrophy and intestinal metaplasia may result from abrasives or irritants in diet (e.g., hard grains, salt, surfactants). Increased pH allows bacterial growth, which increases reduction of nitrate to nitrite. Nitroso compounds formed are mutagenic-carcinogenic. Water supplies in high risk towns reported to contain high concentrations of nitrate.	Correa et al., 1975b
Review.	Early life exposure critical for stomach cancer. Reference to two histological types, precursor lesions, and nitroso compounds as candidate carcinogens. Epidemiological features summarized.	Haenszel and Correa, 1975

Methods	Results and Interpretation	References
<p>Description of registry. Study (in progress) uses questionnaire, chemical tests, gastroscopy, and gastric biopsy of charity hospital patients, and correlations with nitrate in drinking water.</p>	<p>Colombia has a high stomach cancer mortality rate. Immigrants from rural, high-altitude areas of Colombia have highest rates. Intestinal metaplasia and atrophic gastritis are also common in this group. Water in areas with highest stomach cancer rates has high nitrate content.</p>	<p>Correa et al., 1975a</p>
<p>Case-control study in Narino, Colombia, including 276 stomach cancer patients and 276 matched controls from four hospitals. Also, 463 gastroscopies of healthy persons, and 173 water sources were tested for nitrate. Urine and saliva were tested for nitrate and nitrite.</p>	<p>Fourfold geographic variation in stomach cancer shown by case-control studies. Geographic correlation with chronic atrophic gastritis and intestinal metaplasia, nitrate content of well water, and nitrate in urine and saliva. Nitrate consumption by stomach cancer patients and controls not studied.</p>	<p>Cuello et al., 1976</p>
<p>Epidemiological questionnaire sent to 463 volunteers who had gastroscopy.</p>	<p>Atrophic gastritis and intestinal metaplasia were associated with history of corn ingestion and were inversely associated with lettuce.</p>	<p>Haenszel et al., 1976a</p>
<p>Subjects included 586 volunteers from areas of high and low risk for stomach cancer who had gastroscopy. A mathematical model related transition of "precursor lesions"</p>	<p>Seventy-five percent of high-risk population has superficial gastritis or more superficial gastritis or more advanced "precursor" by age 25 years, in contrast to low-risk population.</p>	<p>Correa et al., 1976</p>

Methods	Results and Interpretation	References
Mutagenesis of nitrite plus spermidine tested.	Spermidine nitrosation yields mutagen most active at pH 3.5-6.0, the pH present with hypochlorhydria from "precursor" lesions. Ascorbic acid inhibited mutagenesis.	Correa et al., 1978
Gastric fluid of persons with "precursor lesions" tested for pH, nitrite, nitrate, thiocyanate, and chlorides.	Above pH 5, nitrite was correlated with nitrate. This is compatible with bacterial reduction of nitrate in persons with "precursor" lesions.	Tannenbaum et al., 1979
Similar to those for study described immediately above.	Possible role of N-nitroso compounds discussed.	Correa et al., 1979
Pathology review of biopsy materials.	Gastric dysplasias described. Correlations with gastric juice nitrite noted.	Cuello et al., 1979
Review and discussion.	Two gastric cancer models involving nitrite discussed--one in normal acidic stomach, the other in achlorhydria.	Tannenbaum et al., 1977

TABLE 9-4

Gastric Cancer in Chile

Methods	Results and Interpretation	Reference
Stepwise multiple regression analysis of gastric cancer mortality in 1960 in association with tons of fertilizer used per province, rainfall, and latitude during 1960-1961.	Mortality significantly associated with fertilizer use. Stomach cancer deaths had borderline association with proportion of farmers in each province but no association with proportion of miners.	Zaldívar and Robinson, 1973
Same as above. Repeated using 1960, 1962, and 1964 deaths.	Further association of gastric cancer deaths and use of nitrate fertilizers by province.	Zaldívar and Wetterstrand, 1975
Same as above. Repeated using 1960-1964 nitrate use by province; regional and national trends.	Association still significant.	Zaldívar, 1977

Methods	Results and Interpretation	References
<p>Statistical correlations of gastric cancer mortality in 1957-1972 in association with nitrate in drinking water and nitrogen fertilizers used per province.</p>	<p>No association with nitrate in drinking water, which contains much less than WHO maximum acceptable level. High correlation between estimated per capita nitrogen exposure and gastric cancer.</p>	<p>Armijo and Coulson, 1975</p>
<p>Gastric cancer cases from gastroenterology clinics of seven Santiago hospitals and 845 controls interviewed, 1977-1978.</p>	<p>Cases had long-term residence in high-risk areas in early life; controls had long-term residence in low-risk areas. Association found between stomach cancer and previous occupation in agriculture.</p>	<p>Armijo et al., 1981b</p>
<p>Nitrate levels in urine and nitrite in saliva determined in 231 11- to 13-year-old school-children in two areas with high and two with low stomach cancer mortality in Chile. Levels of nitrate and nitrite in selected vegetables were also tested in high and low rate areas.</p>	<p>Children in low mortality areas had higher urinary nitrate. Salivary nitrite was similar in high and low mortality areas. No overall correlation was seen between nitrate levels in vegetables and mortality rates for stomach cancer.</p>	<p>Armijo et al., 1981a</p>

commonest form of cancer in males during the 1920's and 1930's. Although the incidence rate of this form of cancer has dropped by two-thirds since then, there are still many cases reported each year. The American Cancer Society (1980) has estimated that 23,000 new cases can be expected in 1981. Weisburger and Raineri (1975) speculated that the decline in the incidence of stomach cancer in the United States is due to the introduction of refrigeration, which slows down bacterial contamination of food and, hence, inhibits the production of nitrite from nitrate. Other possible contributing factors could include the increase in the year-round consumption of fruits and vegetables (which contain protective factors such as antioxidants), and the decreased use of varying levels of nitrite to preserve products in the home in favor of better regulated, industry-prepared products. These suggestions, although interesting and supportive of the hypothesis that nitrate, nitrite and N-nitroso compounds may be causative agents in gastric cancer, have not been investigated to determine their validity. One possible exception is the finding of an inverse correlation between consumption of vitamin C-containing fruits and vegetables and the risk of developing gastric cancer (Bjelke, 1978).

Geleperin et al. (1976) compared cancer mortality rates in three Illinois communities whose water supplies contained different levels of nitrate and found no significant correlations. However, few details about the cancer patients or the communities were provided in this report. Higginson (1966) compared diets of 93 stomach cancer patients to controls. Stomach cancer patients had a history of a slightly higher consumption of pork, ham, sausage, and bacon and a lower consumption of dairy products and fresh fruits. However, the differences between the patients and the controls were not statistically significant. Bjelke (1978) also reported a negative association between consumption of fresh fruits containing vitamin C and gastric cancer patients in Norway and the north-central region of the United States.

The Netherlands. Meinsma (1964) interviewed 340 stomach cancer patients and a comparable group of other hospital patients in the Netherlands. The stomach cancer patients of both sexes ate bacon more frequently than did their controls, but the bacon consumption of female patients was lower than that of the male controls. Meinsma also noted that the intake of citrus fruits by cases was lower than that of controls.

In a laboratory study to investigate the possibility that stomach cancer is associated with bacon consumption, Mirvish et al. (1980a) observed that methylurea was produced when fried

and vegetables, meat, or eggs, but do consume large amounts of salted and pickled vegetables and sweet potatoes.

Esophageal Cancer

Esophageal cancer is especially common in parts of Iran, China, the USSR, and South Africa. Epidemiological studies conducted in several of these high incidence areas are described below.

Iran. The high incidence of esophageal cancer in the Caspian Littoral of Iran has been studied by a Joint Iran-International Agency for Research on Cancer Study Group (1977). Villages with different rates of esophageal cancer were surveyed for dietary, work, and personal habits. Their main foodstuffs were analyzed for volatile nitrosamines, nitrate, nitrite, and other compounds, and their drinking water was tested for nitrate and nitrite.

The average daily intake of nitrate and nitrite was not significantly different for high- and low-incidence areas. For example, a comparison of the nitrate and nitrite content of water showed no elevation in high-risk areas. In contrast to these findings concerning nitrate and nitrite in water, Eisenbrand et al. (1980) stated that there were intermittently high levels of nitrite in the saliva of children in the high-incidence area of Iran, especially on hot days when water intake may not have been sufficient.

The study group also found that in the regions where esophageal cancer was most common, the diet was limited largely to bread and tea, and was low in vitamin C, vitamin A, and riboflavin. In a subsequent case-control study in this region, Cook-Mozaffari et al. (1979) also found lower intakes of uncooked vegetables (as well as fruits) in cases compared with controls. However, no association was found between the intake of preformed volatile nitrosamines and high-risk of esophageal cancer (Joint Iran-International Agency for Research on Cancer Study Group, 1977). The results of this investigation did not implicate nitrate, nitrite, or N-nitroso compounds, but indicated that the high esophageal cancer risk in the Caspian Littoral of Iran "arises from the severely limited and probably irritant nature of the diet, in conjunction with exposure to a carcinogenic agent derived either from the opium tars or from wheat contaminants."

Table 9-5 summarizes the major results and conclusions of the epidemiological studies of esophageal cancer in Iran.

TABLE 9-5

Esophageal Cancer in Iran

Study	Methods	Results and Interpretations	References
Esophageal cancer in the Littoral; results; nitrosamine	Villages with varying esophageal cancer incidence were sampled. A census was taken; households surveyed about diet, work, and personal habits; medical exams given to a subsample; main foodstuffs analyzed for volatile nitrosamines, nitrate, nitrite, and other chemicals; and water tested for nitrite and nitrate.	Average daily intakes of nitrate and nitrite were similar for high- and low-incidence areas. Preformed volatile nitrosamines did not appear to be important. The diet in the highest incidence area was markedly restricted to bread and tea and was low in vitamin C, vitamin A, and riboflavin. The investigators tentatively concluded that the high esophageal cancer risk "arises from the severely limited and probably irritant nature of the diet, in conjunction with exposure to a carcinogenic agent derived either from the opium tars or from wheat contaminants."	Joint Iran-International Agency for Research on Cancer Group, 1977
Esophageal cancer in the Littoral; results; case-control	Epidemiological interviews of 344 cases (of 638 registered) and matched controls.	Strong association with low socioeconomic status and low intake of fruits and vegetables.	Cook-Mozaffari et al., 1977

and suggested that nitrosamines are likely to be the causative agents. The incidence of esophageal cancer is highest in Lin-Xien county in Henan province, where there is also a high prevalence of esophageal dysplasia. The occurrence of squamous cell carcinoma in the gullet (esophagus) of domestic chickens in the same area is intriguing. The diet and gastric juice of noncancer patients have been found to contain secondary amines, nitrate, nitrite, and nitrosamines. In laboratory experiments, treatment of a fungus-infected corn bread (eaten in a high-incidence area) with nitrite produced nitrosamines, which may be esophageal carcinogens in rats and possibly in humans (Chuan et al., in press; Li et al., 1980). The diet of this population includes nitrite-rich pickled vegetables and is low in riboflavin and ascorbic acid. The Chinese have begun a preventive effort, discouraging the use of pickled vegetables and fungus-infected corn bread and encouraging the consumption of foods containing ascorbic acid.

The diets of populations in areas with a high incidence of esophageal cancer mortality are similar but not identical to those with high mortality rates from stomach cancer (see section on stomach cancer, above).

United States. The American Cancer Society (1980) has estimated that there will be 8,800 new cases of esophageal cancer in the United States during 1981. There has been no significant change in the rate of this type of cancer in recent years. However, the incidence is rising among the black population.

Esophageal cancer in the United States has been associated with the use of tobacco, which can contain tobacco-specific nitrosamines in concentrations as high as 88 mg/kg. Cancer at this site could be attributable to exposure to carcinogenic tobacco-specific nitrosamines, especially nitrosonornicotine (NNN), which induces esophageal cancer in rats when administered in the drinking water (Hoffmann et al., in press). Cigarette smoke contains other carcinogens that could be involved as well. Furthermore, Wynder and Bross (1961) have suggested that alcohol may promote the carcinogenic effect of tobacco at this site. Additional evidence for the role of nitrosamines in esophageal cancer has been reported by Mettlin et al. (1980), who computed indices for the intake of vitamins A and C in a study of male esophageal cancer patients and controls and found an inverse correlation between intake of these vitamins, especially vitamin C, and cancer risk.

[e.g., nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA) at low doses (Druckrey et al., 1967) and nitrosomethylamylamine (Bulay and Mirvish, 1979) at higher doses] induce tumors of the nasal cavity in rats.

Nasopharyngeal cancer is rare in the United States, but is common among the Chinese in parts of Southeast Asia, including Hong Kong and Singapore. It may be relevant that NDMA has been detected in salted fish from Hong Kong, although the levels were only 1-35 $\mu\text{g/kg}$ (Huang et al., 1978a). When tested in animals, the same fish product induced tumors of the nasal cavity in 4 of 20 rats (Huang et al., 1978b). Other factors in addition to N-nitroso compounds may also be important in the etiology of this disease in humans, including infection with the Epstein-Barr virus (EBV) (Zur Hausen, 1976).

Liver Cancer

In China, the geographic pattern of liver cancer incidence is different from that for cancers of the stomach or esophagus. High levels of nitrate and nitrite in the soil reportedly correlate with increased liver cancer mortality, and nitrosamines have been detected in salted vegetables, which are commonly eaten in areas with high rates of liver cancer (Armstrong, 1980). However, other agents such as aflatoxin and hepatitis B virus have also been implicated as etiologic agents in this disease (Armstrong, 1980).

Bladder Cancer

Some investigators have reported that volatile nitrosamines are present in the urine of healthy volunteers (El-Merzabani et al., 1979; Hicks et al., 1977; Kakizoe et al., 1979); however, when all the necessary controls for artifact formation are implemented, e.g., the use of marker nitrosamines, excess vitamin C, etc. (see Chapter 7), volatile nitrosamines are not found in analytically significant amounts in the urine of healthy volunteers (Archer, personal communication; Eisenbrand et al., 1981; Tannenbaum, 1981).

Dimethylamine is the principal secondary amine in urine and can be present at levels of approximately 0.5 mM (Asatoor and Simenhoff, 1965). Brooks et al. (1972) and Radomski et al. (1978) have reported that high levels of NDMA are present in the urine of patients with urinary tract infections. Although the analytical methods used by both groups of investigators did not include appropriate detection

In an epidemiological study by Wynder et al. (1963), 6% of a group of men with bladder cancer had a history of cystitis -- a significantly higher percentage than that of the controls. However, in the general population, bladder cancer predominates in men, whereas urinary tract infections are more common in women. Howe et al. (1980) reported an increased risk of bladder and kidney cancer in persons with a history of bladder infections and kidney stones. Although it is plausible that the formation of N-nitroso compounds in the infected bladder is related to the occurrence of cancer, further epidemiological studies are needed to determine the validity of this hypothesis.

Occupational Studies

In certain occupations, workers are exposed to airborne N-nitroso compounds. The highest exposures have been reported for leather tanners, who are exposed to NDMA (Fine, 1980a,b; Rounbehler et al., 1979), and rubber-industry workers, who are exposed to N-nitrosomorpholine (NMOR) and NDMA (Fajen et al., 1979; Preussmann et al., 1980). Several epidemiological studies have been conducted to examine the incidence of cancer among rubber-industry workers.

Monson and Nakano (1976) reported increases in certain cancers among rubber-industry workers in specific jobs: cancer of the stomach and large intestine in processers, lung cancer in tire curers, brain tumors and lymphatic cancer in tire builders, and bladder cancer and leukemia in all workers. In other studies of these workers, Mancuso et al. (1968) reported an increase in brain tumors, while McMichael et al. (1974) found high mortality ratios for leukemia and cancer of the stomach and prostate, and low ratios for cancer of the bladder, lung, brain, and pancreas. The data from these epidemiological studies are not very consistent, and the exposure to N-nitroso compounds was not specifically examined. Moreover, comparisons of exposures may be misleading because the purity of chemicals used in different factories within the same country and in different countries varies greatly.

Some other occupational groups that might be exposed to nitrosamines include foundry workers (Egan et al., 1979), agricultural workers who use pesticides (Bontoyan et al., 1979), workers in chemical plants manufacturing amines, and, perhaps, brewery workers (Walker et al., 1979). Although no studies of nitrate and/or nitrite exposures of workers have been reported, butchers and meat cutters have been shown to have a high incidence of prostate cancers (Registrar-General for England and Wales, 1978).

Studies in Animals

This section reviews the results of studies conducted in animals to investigate the carcinogenicity of nitrate, nitrite, or N-nitroso compounds.

Nitrate

The few experiments conducted in animals have provided no evidence that nitrate is carcinogenic. Greenblatt and Mirvish (1973) gave sodium nitrate in distilled water (12,300 mg/liter) to 40 Strain A mice as a substitute for drinking water. An equal number of mice served as controls for this and other experiments. The animals were treated for 25 weeks, and the experiment was terminated 13 weeks later. The number of lung tumors in treated and control mice was similar. Lijinsky et al. (1973) administered 40 mg of sodium nitrate daily in drinking water to each of 15 male and 15 female MRC-derived rats for 84 weeks and terminated the experiment 20 weeks later. An increase in pituitary adenomas was observed in treated female rats, but this was not statistically significant. There were no other tumors that could be associated with the treatment. Sugiyama et al. (1979) reported another experiment in which there was no significant difference in the incidence of tumors among the controls and ICR mice fed 50,000 and 25,000 mg of sodium nitrate per kilogram of diet over a lifetime.

Nitrite

There have been very few adequate studies to test the carcinogenicity of nitrite in animals and, until recently, most of the information came from data on tumor incidence in control animals administered nitrite alone in experiments that were designed to study the carcinogenic effects resulting from the simultaneous administration of nitrite and an amine.

In one recent study, which was designed to test the carcinogenic effect of nitrite, Mirvish et al. (1980a) reported that papillomas of the forestomach developed in 8 of 45 MRC Wistar rats (of both sexes) given a 3,000 mg/liter solution of sodium nitrite in distilled drinking water for 5 days/week for life (more than 100 weeks). Two of 91 untreated rats developed these tumors. This significant increase in the yield of benign tumors was produced by the maximum tolerated

of hemangioendothelial cells. However, because NDMA and nitrosopyrrolidine were found in the diet containing sodium nitrite, the author concluded that these N-nitroso compounds were probably the principal cause of the liver tumors in this experiment.

Inai et al. (1979) fed sodium nitrite at 5,000, 2,500, and 1,000 mg/liter in drinking water to groups of 50 male and 50 female ICR mice for 18 months. No tumors attributable to nitrite treatment were observed. In a cocarcinogenesis assay of morpholine plus sodium nitrite, Shank and Newberne (1976) reported that control Sprague-Dawley rats fed sodium nitrite at a level of 1,000 mg/kg in the diet led to a 27% incidence of tumors of the lymphoreticular system compared to approximately 6% in untreated control rats.

In another, larger lifetime study conducted for the Food and Drug Administration (FDA), Newberne (1978, 1979) administered sodium nitrite to groups of approximately 68 male and 68 female Sprague-Dawley rats under a variety of conditions. Groups 1 to 5 received 0, 250, 500, 1,000, or 2,000 mg/kg sodium nitrite in the diet, and groups 6 and 7 received 1,000 or 2,000 mg/liter in drinking water. For these groups, an agar-based semisynthetic diet was used. For groups 9 to 11, a commercial chow diet was substituted, and sodium nitrite concentrations of 0, 1,000, or 2,000 mg/kg diet were fed to the animals. Groups 13 and 14 were given a refined casein diet containing nitrite at 0 or 1,000 mg/kg, while another two groups, 15 and 16, were fed the original semisynthetic diet containing nitrite at 0 or 1,000 mg/kg. Each of the latter two groups contained only 34 animals -- the dams that supplied the pups for groups 1 and 4. Groups 17 and 18 were also fed the semisynthetic diet containing nitrite at 0 or 1,000 mg/kg. Groups 1 through 16 were exposed prenatally, while groups 17 and 18 were exposed at 21 days. Groups 8 and 12 served as positive controls and received urethane (2,000 mg/liter) in drinking water or the semisynthetic diet, respectively. The rats survived the sodium nitrite regimens well, the only adverse effects being a loss of weight in groups receiving 2,000 mg/kg in their diet and, to a lesser extent, in groups receiving 2,000 mg/liter in drinking water.

Newberne's histopathologic assessment of the tissues indicated that by considering all the groups receiving sodium nitrite together there was a statistically significant excess of lymphoid tumors ($p < 0.01$, based on chi-square analysis). This was reflected especially in the groups receiving sodium nitrite in drinking water, where the excess of lymphoid tumors was statistically significant, but the results were not significant in the other groups treated with sodium nitrite.

cell proliferation in the spleen and, occasionally, in the lymph nodes of some members of all groups except the positive controls (groups 8 and 12). The incidence of this abnormality in nitrite-treated animals, however, was greater (11.2%) than in the untreated animals (7%). The disease in humans, which is histologically similar to that observed in rats, is considered by some to develop into lymphoma; others consider it not to be preneoplastic.

Newberne interpreted these results to indicate that nitrite is an enhancer or promoter of carcinogenesis in rats.

After Newberne's report was submitted, a Government Interagency Working Group on Nitrite Research reviewed a sample of histological slides from that study and decided that there was sufficient difference of opinion in the diagnoses to warrant a further evaluation of the histopathological findings. The Universities Associated for Research and Education in Pathology (UAREP), a nonprofit consortium of 15 universities organized to carry out educational and research activities in pathology, was selected by the FDA to review the slides (Food and Drug Administration, 1980a). A Joint Committee of Experts, which was established by the UAREP to perform this review, diagnosed fewer lymphomas than Newberne had reported (Food and Drug Administration, 1980a). The disparity between the two series of diagnoses involved the differentiation of lymphomas from extramedullary hematopoiesis, plasmacytosis, or histiocytic sarcoma. Furthermore, the committee was unable to confirm Newberne's diagnosis of immunoblastic hyperplasia.

In its final report to the FDA, the Government Interagency Working Group summarized its assessment of the UAREP committee's findings as follows:

"The major result of the histopathology review was that most of the lymphoma diagnoses originally reported were not confirmed. A relatively high incidence of lymphomas had been reported by Dr. Newberne, with a significant increased incidence in the total combined treated groups as compared to combined controls. The UAREP pathologists, on the other hand, diagnosed very few lesions as lymphoma, with a resulting reduction of incidence to approximately 1 percent among treated and control groups. This rate of lymphoma incidence is similar to that usually seen spontaneously in Sprague-Dawley rats.

histiocytic sarcomas, angiosarcomas, liver neoplasms, ear duct tumors, pancreatic tumors, pituitary tumors, and mammary tumors. However, after statistical analysis and careful review by the IAWG, no demonstration could be found that the increased incidences of these tumors were induced by the ingestion of sodium nitrite." (Food and Drug Administration, 1980b, pp. 28-29)

The committee has reviewed the data in 21 additional studies identified by Birdsall (1981) in which the carcinogenicity of nitrite could be examined. In the committee's view, three of the 21 reports were too brief for an adequate evaluation (Olsen and Meyer, 1977; Pearson et al., 1980; Procter and Rona, 1977). Of the remaining 18 studies, nine were conducted in rats, eight in mice, and one in guinea pigs. The experimental designs of these studies varied greatly and the end points for carcinogenicity in some of them were specific lesions. For example, the incidence of pulmonary adenomas was the carcinogenic "end point" in four studies.

In two relatively short-term studies (7.5 to 12 months), nitrite was administered to pregnant Swiss mice intragastrically (10 mg/kg body weight) each day during the last week of pregnancy (Börzsönyi et al., 1978) or in drinking water (500 mg/liter during the entire pregnancy (Börzsönyi et al., 1976). In a third study, nitrite was given to rats in drinking water (100 mg/kg body weight) for three generations (Druckrey et al., 1962b). In a fourth study, nitrite was injected subcutaneously (12 mg per mouse over 90 days), and the experiment was concluded at 11 months (Nakahara and Fukuoka, 1959).

In another four experiments, nitrite was fed in doses of 1,600 or 2,000 mg/kg, and the experiments were terminated at 16 (Matsukura et al., 1977), 29 (Lijinsky and Reuber, 1980; Van Logten et al., 1972), or 32 (Taylor and Lijinsky, 1975) months. In 10 studies, nitrite was added to the drinking water for periods varying from 7.5 (Greenblatt and Mirvish, 1973) to 30 months (Greenblatt et al., 1973; Sen et al., 1975). In five of these, the observation period was longer than 24 months (Garcia and Lijinsky, 1973; Greenblatt et al., 1973; Lijinsky et al., 1973, 1980; Sen et al., 1975). None of these studies indicated that nitrite was carcinogenic; however, many of them were not designed to test nitrite, and some of them do not meet accepted standards for the adequate assessment of the carcinogenicity of chemicals (Interagency Regulatory Liaison Group, 1979).

may interact with specific dietary components or endogenous metabolites to produce N-nitroso compounds that induce cancer. The initial reports on this subject suggested that ingested nitrite could react with secondary amines in the acidic conditions of the stomach to produce nitrosamines that induce tumors (Sander, 1970; Sander and Bürkle, 1969). Three main lines of research have developed since these first studies were reported. First, many experiments in animals were performed to confirm and amplify this novel observation (Greenblatt and Mirvish, 1973; Greenblatt et al., 1971; Mirvish et al., 1972a; Shank and Newberne, 1976). Second, the kinetics of nitrosation were intensively studied to determine the rate of carcinogen formation for different reactants. Third, attempts were made to measure the amounts of N-nitroso compounds formed under defined conditions (Mirvish, 1975; Mirvish and Chu, 1973; Mirvish et al., 1973, 1980b).

The second and third areas of research are particularly important since many entities are able to react with nitrite: some of them react readily, while others react very slowly (Chapter 4), and only some of these reactions will lead to the formation of carcinogenic N-nitroso compounds and the subsequent development of tumors. In one study, for example, Greenblatt and Mirvish (1973) reported that the kinetics of in vivo nitrosation of piperazine, as evidenced by tumor formation, closely resembled those occurring in vitro. They reported that the incidence of lung tumors in Strain A mice exposed for 5 to 6 months was directly related to the concentration of piperazine and to the square of the nitrite concentration.

Because the tumorigenic effect is very dependent on the dose of nitrite and nitrosatable substrate, caution must be exercised when using nitrosation data from laboratory experiments to predict the response of humans exposed to nitrite and nitrosatable agents. Nitrosation experiments designed to produce a measurable incidence of tumors are generally carried out with very high levels of nitrite and nitrosatable agents. With the possible exception of exposure to nitrosatable drugs, humans are generally exposed to much lower levels of these agents. Since the concentrations of both nitrite and nitrosatable substances are related to the final concentration of the N-nitroso compound formed, extrapolation of this information probably means that the carcinogenic response in the human population is likely to be less than that observed in experiments on laboratory animals.

For example, according to chemical kinetic equations, lowering the nitrite concentration 100-fold, from 10,000 mg/liter to 100 mg/liter, and lowering concentrations of the nitrosatable agent by an equal factor will lower the concentration of the N-nitroso compound

reaction of nitrite or nitrogen oxides with nitrosatable substances.

N-Nitroso Compounds

Chronic Exposure Experiments. Shortly after NDMA was introduced as a novel industrial solvent, severe and, on one occasion, fatal liver damage occurred in humans exposed to the compound. A laboratory investigation of this agent (Barnes and Magee, 1954) confirmed the clinical observations. The investigators found that subchronic treatment with NDMA led to changes in the liver of rats similar to those produced by known liver carcinogens. Carcinogenicity tests on NDMA in rats (Magee and Barnes, 1956, 1959) demonstrated that chronic administration of the chemical for 42 or 102-104 weeks at low levels (10, 20, 50 mg/kg diet) led to liver cancer, whereas shorter exposures (1 to 4 weeks) at higher doses (100-200 mg/kg diet) induced kidney tumors.

Following this initial demonstration, numerous carcinogenicity studies on variety of N-nitroso compounds have been reported. Druckrey and his colleagues (1967) exposed rats to 65 N-nitroso compounds, most of which were potent carcinogens. Lijinsky and Reuber (1981) examined many other N-nitroso compounds for their carcinogenic potential, mainly in rats. Of the approximately 300 N-nitroso compounds tested to date, 85% of the 209 nitrosamines and 92% of the 86 nitrosamides have been shown to induce cancer in laboratory animals (Preussmann and Stewart, personal communication, 1981). Schmähl et al. (1978) reported in a review that NDEA was carcinogenic in 20 species of animals. Among the species in which N-nitroso compounds have been shown to be carcinogenic are rats, mice, guinea pigs, rabbits, dogs, monkeys, grass parakeets, and pigs (Schmähl and Osswald, 1967), hamsters (Dontenwill and Mohr, 1961), hedgehogs (Graw et al., 1974), mink (Koppang and Rimeslätten, 1976), and trout (Halver et al., 1962). N-Nitroso compounds have induced tumors in all species tested to date.

Some of the N-nitroso compounds shown to be carcinogenic in animals have been found in various environments to which humans are exposed (Chapter 7). In addition to NDMA and NDEA, these compounds include: nitrosodiphenylamine (NDPhA) (Cardy et al., 1979), NMOR (Bannasch and Müller, 1964), nitrosodiethanolamine (NDELA) (Druckrey et al., 1967; Lijinsky et al., 1980; Preussmann et al., in press), nitrosopyrrolidine (NPYR) (Druckrey et al., 1967; Greenblatt and Lijinsky, 1972b), nitrosodi-n-propylamine (NDPA) (Druckrey et al., 1967), nitrosodi-n-butylamine (NDBA) (Druckrey et al., 1962a, 1967; Mohr et al., 1970; Takayama and Imaizumi, 1969), NNN (Hoffmann et

Takayama, 1969). (References given for each compound provide carcinogenicity data; See Chapter 7 for discussion of the distribution and concentrations of these nitrosamines in various environmental media.)

N-Nitroso compounds that are not carcinogenic include the nitroso derivatives of some amino acids (Druckrey et al., 1967; Greenblatt and Lijinsky, 1972a; Mirvish et al., 1980a) and certain nitrosamines that do not contain one or more alpha hydrogen atoms on the carbon next to the N-NO group (Druckrey et al., 1967).

One of the important conclusions reached in the many studies of carcinogenic N-nitroso compounds is that different compounds have the ability to induce tumors of specific tissues. Under certain conditions, NDMA and NDEA were carcinogenic in the liver of rats. NDEA, even in low doses, was also carcinogenic in the lungs and/or esophagus of rats and hamsters. NDBA and certain metabolic derivatives were bladder carcinogens in rats and mice (Druckrey et al., 1964; Wood et al., 1970). Unsymmetrical nitrosodialkylamines, such as nitrosomethylamylamine, induced esophageal cancer in rats (Bulay and Mirvish, 1979; Druckrey et al., 1967; Mirvish et al., 1978), whereas MNNG in rats provides a standard animal model for the induction of gastric cancer (Saito and Sugimura, 1973). Nitrosoethylurea, especially when administered transplacentally to rats, led to tumors of the brain and spinal cord (Ivankovic and Preussmann, 1970), whereas peripheral nervous system tumors resulted in hamsters (Rustia and Shubik, 1974). More recently, a group of nitrosamines analogous to, or converted metabolically to, nitrosobis(2-oxopropyl)amine (BOP) or nitrosomethyl-2-oxopropylamine (MOP) were shown to produce ductular cell carcinomas of the pancreas in hamsters (Pour et al., 1974, 1978); however, in other species, such as rats, guinea pigs, or mice, tumors only occurred at sites other than the pancreas.

Thus far, determinations of structure-activity relationships have applied to partial structures that may lead to cancer at selected sites. In fact, because most N-nitroso compounds are carcinogenic, the amount of useful information on structure-activity relationships that may be obtained is limited unless these relationships are derived from quantitative measures of carcinogenicity.

Dose-Response Studies and Carcinogenic Potency. Druckrey (1967) determined the relationship of time to death and tumor incidence as a function of dose for NDEA in BD II rats given a series of daily doses ranging from 0.075 to 14.2 mg/kg body weight (Table 9-6). At each of seven dose levels, ranging from 0.3 to 14.2 mg/kg body weight day, liver carcinomas were induced in every surviving rat, but the time to death ("latency") decreased from 457 to 62 days as the dose increased.

<u>Daily Dosage,</u> <u>mg/kg Body Weight</u>	<u>Yield of Carcinomas/ No. of Survivors</u>	<u>Average Total Dose, mg/kg Body Weight</u>	<u>Average Induction Time, Days</u>
14.2	5/5	1,000	68
9.6	25/25	963	101
4.8	25/25	660	137
2.4	34/34	460	192
1.2	36/36	285	238
0.6	49/49	213	355
0.3	67/67	137	457
0.15	27/30	91	609
0.075	5/7	64	840

^aData adapted from Druckrey, 1967, and Preussmann, 1978.

weight/day), the tumor yield was less than 100% and the induction times were longer. The investigators suggested that these data fit the expression:

$$dt^n = \text{constant},$$

where d is the daily dose, t is the average time to tumor, and n is a constant, which was 2.3 for NDEA and varied between 1.2 and 4.7 for other carcinogens.

Several factors limit the ability to draw definite conclusions from these data. The lowest tumor yield was 71% (five rats with tumors of seven that survived long enough to develop tumors), and there can be no certainty that extrapolation from tumor incidence at high doses to lower doses will be meaningful. Time-to-death rather than time-to-tumor is used, implying either that the time for the induced liver tumors to kill the rats is short or is a relatively constant fraction of the induction time. Such assumptions may or may not be valid, and can only be assessed by experiments involving serial sacrifices. The number of surviving rats, especially those in the highest and lowest dose groups (five and seven, respectively), is insufficient. The importance of this study is that at a particular dose of the tumorigen, time-to-tumor occurrence, as well as the number of rats with tumors, are clearly shown to be of critical importance in quantifying the effects of a carcinogen.

It is unlikely that all of this information will be experimentally determined in animal models for every N-nitroso compound. Hence, there is a need for a simple expression of carcinogenic potency that can be derived from the simplest protocol used in a carcinogenicity study.

Clayson (in press) has described an expression that may give an approximate indication of the potency of an N-nitroso compound:

$$\text{Potency} = 7 - \log d_{E50},$$

where d_{E50} is that dose in $\mu\text{mol/kg}$ body weight per week that will give a 50% tumor yield in a lifetime study, and 7 is an arbitrary constant that ensures all values are positive. Using this expression and appropriate approximations, it is possible to obtain an indication of the potency of any chemical that has been demonstrated to be carcinogenic below the 100% tumor incidence level (Table 9-7). These methods have been used to determine potency values for the N-nitroso compounds shown in Table 9-8. To develop these values, the committee used data reported in the literature in which the incidence of tumors was as close to 50% as possible in order to avoid unnecessary extrapolation. Where necessary, the following assumptions were made:

- Tumor yield and survival. In most carcinogenicity tests, the survival time is less than the normal lifespan of the test animal and the tumor probability differs from 0.50. To correct for this, we assume $d_{E50} = d_x \cdot \frac{0.50}{P_x} \cdot \frac{S_x}{S_L}$, where d_{E50} has the same meaning as in the potency expression, P_x is the probability of obtaining a tumor in an animal at dose rate d_x , S_x is the survival at dose rate d_x , and S_L is the expected lifespan of the animal. These assumptions are approximations that should be acceptable if (1) tumor probability and survival do not differ too greatly from 0.50 and S_L , respectively, and (2) potency is calculated in log units. For example, Druckrey (1967) suggested that for a carcinogen, dt^n is a constant, where d = dose rate and t is time to tumor occurrence. It is assumed here that $n = 1$, although Druckrey et al. (1963) indicated that it may be higher for specific chemicals.

- Dose rate. When a dose of carcinogen was administered for 60% or more of the survival period of the animals, it was assumed that this represented the actual dose rate for the entire experiment.

The approximate potency values shown in Table 9-8 demonstrate that N-nitroso compounds have a wide range of carcinogenicity

The Potency of a Range of Chemicals that are Carcinogenic in Rats
or Mice Following Continuous Feeding^a

<u>Chemical</u>	<u>Species (Tumor Site)</u>	<u>Potency (Log Units)</u>
Aflatoxin	Rat (liver)	9.2
NDEA	Rat (liver)	6.5
Michler's Ketone	Rat (liver)	4.6
Carbon tetrachloride	Rat (liver)	3.9
2-Aminoanthraquinone	Rat (liver)	4.4
Trichloroethylene	Mouse (liver)	2.1
Saccharin	Rat (bladder)	1.9

^aAdapted from a table in Clayson (in press), based on data from selected experiments reported in U.S. Public Health Service, 1951-1978.

TABLE 9-8

Approximate Potency Value for Specific N-Nitroso Compounds^a

<u>Agent</u>	<u>Species</u>	<u>Route</u>	<u>Target Tissue</u>	<u>Potency (Log Units)</u>
NDEA	Rat	Oral	Liver	6.4
NDMA	Rat	Oral	Liver	5.7
Nitroso-1,1-diethyl-3-methylurea	Rat	Oral	Nervous System	4.4
Nitrosotriethylurea	Rat	Oral	Nervous System	4.1
Dimethylnitramine	Rat	Oral	Liver	3.2
NDPhA	Rat	Oral	Bladder	3.2
Nitrosobis(2-ethoxyethyl)amine	Rat	Oral	Liver	2.7

^aPotency was calculated by the committee, based on data from U.S. Public Health Service, 1951-1978.

Table 9-9 shows the variation in the potency of one nitrosamine, NDEA, in six different species. The range is approximately a thousandfold.

The potency values presented in Tables 9-7, 9-8, and 9-9 for a comparison of relative carcinogenicity are crude measures and depend only on one point, the 50th percentile, on the dose-response curve. Other measures of potency depending on lower percentiles or the shape and slope of the dose-response curve may prove to be more relevant if carcinogenicity experiments that have more extensive dose-response curves in the low-dose range are performed. However, the measures for potency given in these tables are useful to illustrate the wide variability in the potency of various carcinogens, especially the N-nitroso compounds.

Potency values are useful in assessing the relative carcinogenicity of various N-nitroso compounds; however, when considering the in vivo formation of N-nitroso compounds, the ease of nitrosation and concentration of the amino substrate will also be important factors. For example, the kinetic rate constants may vary by up to five orders of magnitude for

TABLE 9-9

Carcinogenic Potency of NDEA in Several Species After Continuous Administration^a

Species	Stock	Route	Estimated Lifespan, Months	Tissue of Origin of Tumors	
Rat (<u>Rattus norvegicus</u>)	BD II	Oral	24	Liver	6
White-tailed rat (<u>Myiostomys albicaudatus</u>)	--	Oral	72	Liver	6
Chicken (<u>Gallus domesticus</u>)	White Leghorn	i.m. ^b	100	Liver	5
Guinea pig (<u>Cavia procillus</u>)	Hybrid	Oral	84	Liver	4
	NMRI	Oral	18	Liver	4
Mouse (<u>Mus musculus</u>)	RF	Oral	18	Liver	3
Syrian golden hamster (<u>Mesocricetus</u>)	--	Oral	18	Trachea	3

different N-nitroso compounds (Chapter 4; Mirvish, 1975). This difference, combined with a three orders of magnitude range of potency for the carcinogenicity of these compounds, suggests that certain agents that are readily nitrosated to form more potent carcinogenic N-nitroso compounds (amines or amides) may be of much greater importance in the production of cancer by nitrite than are other agents. It also offers the possibility of determining which nitrosatable agents may be most important in the induction of cancer, even when they are present in small quantities. In addition, information on the relative potency of N-nitroso compounds may aid in studies of chemical structure and carcinogenic activity.

Single Dose Exposure. A single application of certain N-nitroso compounds to adult animals may induce cancer. For example, nitrosomethylurea administered to rats in a single oral dose of 90 mg/kg body weight induced cancers in the kidney, stomach (squamous cell carcinoma), small and large intestine, skin, and jaw (Leaver et al., 1969); NPIP and NMOR led to tracheal and laryngeal cancer in Syrian golden hamsters after a single subcutaneous injection (Althoff et al., 1974); and NDEA resulted in adenomas and carcinomas in the kidneys of rats after a single intravenous injection (Mohr and Hilfrich, 1972).

Induction of kidney tumors by single doses of NDMA has been studied intensively by Hard and his colleagues (Hard, 1979; Hard and Butler, 1970). Magee and Barnes (1962) clearly showed that limited exposure of rats to NDMA led to a high incidence of renal adenomas and carcinomas. The amount of carcinogen that could be applied was limited by liver toxicity, which led to the early death of the rats. However, if the rats were fed a very low ("zero") protein diet for up to 10 days before the single intraperitoneal injection of carcinogen was administered, toxicity in the liver was reduced and higher doses of NDMA could be administered. Under these conditions, a 100% incidence of renal neoplasms was consistently observed (McLean and Magee, 1970; McLean and Verschuuren, 1969; Swann and McLean, 1971).

There is a relative paucity of dose-response data on the effects of single doses of N-nitroso compounds. Mohr and Hilfrich (1972) reported that single doses of NDEA given to female rats at 1.25 mg/kg body weight induced renal adenomas in 10% of the animals. Syrian golden hamsters given a single dose of NDEA in a study by Ii et al. (1979) showed a linear dose-response between 10 and 1.25 mg/kg body weight. Pour et al. (1980) noted that a single injection of nitrosobis(2-xypropyl)amine at 2 g/kg body weight

NDEA at 1.5, 5, and 10 mg/kg body weight injected into 1- or 15-day-old (C57BL/6J X C3HeB/FeJ)F₁ mice induced metastasizing liver tumors. They observed a dose-dependent tumor response to this compound. The highest dose administered to 1- and 15-day-old mice resulted in a 96% and 100% tumor incidence, respectively. Five mg/kg resulted in a 64% and 74% incidence, and 1.5 mg/kg in a 25% and 54% incidence, respectively.

Transplacental Carcinogenesis. The developing organism, i.e., the fetus and neonate, differs from the adult in many ways, several of which are important in the chemical induction of cancer. These differences include metabolic capability, cell proliferation and cell types in certain tissues, hormonal balance, and immunological capacity. The metabolic capability of fetal tissues is generally lower, and often different, than tissues in the adult. In rats, for example, the liver changes from a hematopoietic tissue to its more normal function in the third to the fifth day of extrauterine life. Exposure to trace levels of toxicants such as tetrachlorodibenzo-dioxin (TCDD) during fetal life appears to affect certain metabolizing enzymes for long periods after exposure (Hook et al., 1975; Lucier et al., 1977). At certain stages of development, the nervous system, the liver, and the bladder undergo rapid cell division in contrast to the absence, or very low levels, of cell division later in life. Also, because the immune system does not fully develop until after birth, the fetus and the neonate depend on maternal antibodies for immunologic protection.

The differences in metabolic capability are especially important when considering transplacental carcinogenesis of nitrosamines. Nitrosamides, nitrosoureas, nitrosocarbamates, and nitrosoguanidines generally decompose spontaneously to their ultimate carcinogenic form (Miller and Miller, 1976). Nitrosamines require metabolism, which, it is believed, consists of hydroxylation to an α -hydroxynitrosamine (Chapter 8). The product then decomposes to a carcinogenic electrophile.

The pattern of response of the developing organism to chemical carcinogens is therefore different from that of adults (Tomatis, 1979). The first observation supporting this conclusion was made by Druckrey et al. (1966), who administered nitrosoethylurea at 80 mg/kg body weight to three female BD IX rats on day 15 of pregnancy. Five rats from one litter survived for 160 or more days and developed, or showed signs of, intracranial tumors. In a second experiment, Ivankovic and Druckrey (1968) demonstrated that nitrosoethylurea administered to pregnant Sprague-Dawley rats at concentrations of 1 to 50 mg/kg body weight led to the development of brain and spinal cord tumors in 16% to 100% of the offspring. These tumors were also induced in

developed in less than 10 weeks (Diwan and Meier, 1974). In rabbits given nitrosoethylurea at 60 mg/kg body weight, 14 of 15 animals developed kidney tumors after a mean latency period of 3.5 months, a very short time to tumor occurrence in this species (Fox et al., 1975). In Syrian golden hamsters treated with ethylurea and sodium nitrite (which react in the acidic conditions in the stomach to give nitrosoethylurea), Rustia and Shubik (1974) observed tumors of the peripheral nervous system instead of the tumors of the central nervous system induced in rats. These were more prevalent and had a shorter latency period in females than in males, thereby providing a useful model for neurofibromatosis (von Recklinghausen's disease) in humans.

Other nitrosamides and nitrosoureas given transplacentally to rats or mice have proved to be carcinogenic. These include nitroso-methylurea (Napalkov, 1973; Tomatis et al., 1975), nitrosoethylbiuret (Druckrey and Landschütz, 1971), and nitrosomethylurethane (Tanaka, 1973). Each of these well-designed experiments demonstrated that these compounds have considerable carcinogenic activity.

Transplacental administration of nitrosamines provided much less adequate evidence for carcinogenicity. The best comparative studies in this area were reported by Althoff and Grandjean (1979), who studied the carcinogenesis of 10 nitrosamines in Syrian golden hamsters. In each case, the dams that were treated during pregnancy were maintained so that there could be a direct comparison between the effects of a single dose in the mothers and those in the offspring. The investigators ascertained that the nitrosamine reached the fetal circulation. The chemicals investigated were NDMA, NDPA, NDBA, NPiP, nitrosohexamethyleneamine, nitroso-2-hydroxypropylpropylamine, nitroso-2-oxopropylpropylamine, nitroso-di(2-hydroxypropyl)amine, nitroso-4-hydroxybutylbutylamine, and nitrosomethylpropylamine. Overall, the low tumor yields were remarkably similar in the dams and the female offspring. The major overall difference was a greater sensitivity of the dams to respiratory tract tumors in response to 7 of the 10 chemicals investigated. This experiment clearly demonstrated that the offspring are not exquisitely sensitive to the carcinogenic effects of nitrosamines. The probable explanation of this observation is that nitrosamines require metabolic activation and the fetus does not necessarily possess the requisite enzymes for this purpose.

Although the fetus is not highly sensitive to the carcinogenicity of nitrosamines, neonates, in some instances, are extremely sensitive to their carcinogenic effects. For example, Rao and Vesselinovitch (1973) found that the rate at which hepatomas developed in mice injected

with NDEA at 48 days of age lagged far behind the development of tumors in mice injected with the same dose at 15 days of age. This finding and others reviewed by Craddock (1976) suggest that cell proliferation may play an important role in carcinogenesis mediated by N-nitroso compounds.

Inhibitors or Enhancers of Carcinogenesis. A variety of agents either enhance or inhibit the carcinogenicity of N-nitroso compounds. Such agents may influence the amounts of N-nitroso compounds formed by in vivo nitrosation or may either act on the process of carcinogenesis induction by preformed N-nitroso compounds. These two aspects must be considered separately.

Inhibition of the carcinogenicity of several N-nitroso compounds has been observed in systems where the formation of N-nitroso compounds has been inhibited. Following the initial demonstration by Mirvish et al. (1972b) that ascorbic acid (vitamin C) prevented oxytetracycline from producing NDMA in the presence of nitrite, many experiments were conducted in a variety of animal species with different nitrosatable agents. These studies have clearly demonstrated the inhibition of in vivo nitrosation reactions by ascorbic acid (Akin and Wasserman, 1975; Archer et al., 1975; Fong and Chan, 1976; Greenblatt, 1973; Ivankovic et al., 1975; Mirvish et al., 1975; Rustia, 1975). The principle behind nitrosation inhibition is that ascorbic acid and a variety of other agents compete with the nitrosatable agent for the available nitrite in the acid conditions of the stomach, thereby inhibiting the formation of N-nitroso compounds. A number of other agents that interact readily with nitrite have also been shown to inhibit nitrosation. Among these are other isomers of ascorbic acid, some phenols, and α -tocopherol. Most of these interactions have been observed at the chemical rather than at the biological level. These interactions are described in Chapters 4 and 6.

The formation of N-nitroso compounds can also be enhanced by a variety of ions, especially thiocyanate and iodide, which may catalyze the nitrosation reaction in the stomach (Boyland and Walker, 1975; Boyland et al., 1971; Lathia and Rütten, 1979; Mirvish et al., 1975). Since these ions are present in foodstuffs, their catalytic action could be of some importance in assessing the risk of nitrosation in vivo. The majority of the information on this mechanism has been obtained in vitro using chemically defined conditions, although a recent report by Pignatelli et al. (1981) showed that certain phenolic catalysts enhanced in vivo formation

in the liver and raising the dose that kills 50% of the animals (LD_{50}). The higher doses that may be given with this protein-deficient diet lead to a 100% incidence of induced kidney tumors. In studies in rats, Rogers et al. (1974) showed that diets deficient in lipotropic agents such as choline and methionine enhanced hepatocarcinogenesis by NDEA and NDBA, but not that induced by NDMA. They also enhanced the induction of esophageal tumors by NDBA.

Overall, however, the effort directed toward the study of factors that may affect the metabolism of nitrosamines appears to have been much less extensive than that directed toward other classes of carcinogens, perhaps because most metabolic studies have been conducted on compounds with relatively simple structures, such as NDMA or NDEA (e.g., Phillips et al., 1975). Thus, there have been few bioassays for carcinogenicity using combinations of agents that may influence metabolic activation in combination with carcinogenic nitrosamines. For example, 3-methylcholanthrene inhibits liver tumorigenesis when administered with 3-methyl-4-dimethylaminoazobenzene, a carcinogenic aminoazo dye (Richardson et al., 1952), but does not have a major effect on the induction of liver tumors by NDMA or NDEA (Hoch-Ligeti et al., 1968; Makiura et al., 1973). The former, but not the latter, authors reported that cofeeding 3-methylcholanthrene and NDMA marginally increased the yield of subcutaneous sarcomas induced by 3-methylcholanthrene alone. Schmähl and von Stackelberg (1968) failed to observe any effect of lactoflavin, nicotinamide, or dipyridamole on rat liver carcinogenesis induced by NDEA.

Enhancement of carcinogenicity by N-nitroso compounds can also occur following metabolic activation, at the stages of tumor initiation or promotion. The concept of multistage carcinogenesis has been well established by clinical observations and animal experimentation (Farber and Cameron, 1980). It appears that many, if not all, neoplasms arise from precursor lesions through a series of steps, during which they acquire increasing degrees of autonomy (Foulds, 1958; Medina, 1975). This process is called tumor progression. The two general stages in the progression of tumors are initiation and promotion.

Tumor initiation refers to the earliest irreversible effect of exposure to a carcinogen. This effect might be a consequence of somatic mutation, and it may not be associated with any recognizable phenotypic changes. One of the most important factors altering the magnitude of tumor initiation is the rate of cell replication in tissues that are at risk of carcinogenesis. This is probably best exemplified by the increased incidence of hepatic neoplasms in newborn and suckling animals (in comparison to adults) that have been

in a number of instances when carcinogens were administered in single doses during the phase of most rapid DNA synthesis within one day after partial hepatectomy (Craddock, 1976). Thus, enhancement at the initiation stage requires that the carcinogen be administered after the cells have been stimulated to multiply.

Promotion, on the other hand, refers to a process that enhances or fosters further development of neoplasms from the initiated state through a series of potentially reversible but recognizable stages to a stage of self-sustained neoplasia (Berenblum and Shubik, 1947; Boutwell, 1974). In this case, then, the carcinogen is administered first, followed by the promoting agent. Pure tumor promoters are agents that by themselves have little or no carcinogenic effect, but that greatly increase the incidence of neoplasms when administered after an initiator or low dose of a complete carcinogen. Tumor promotion has been observed in at least nine different organ systems in rats, mice, and dogs (Pitot and Sirica, 1980). Phenobarbital has been found to be a promoter of NDMA-induced carcinogenesis in the rat liver (Pitot et al., 1978), and lithocholic acid was reported to be a promoter of carcinogenesis induced by MNNG in the rat colon (Lipkin, 1975; Reddy and Watanabe, 1979).

Probably the best documented modification of the carcinogenicity of N-nitroso compounds is the enhancement of NDMA- or NDEA-induced liver carcinogenesis in rats and mice by carbon tetrachloride (Pound et al., 1973a; Schmähl et al., 1965; Taylor et al., 1974). Pound et al. (1973a) gave the animals a single necrotizing dose of carbon tetrachloride, followed by a single dose of NDMA 42 or 60 hours later. When administered separately, carbon tetrachloride did not induce liver tumors under these conditions, and NDMA (20 mg/kg body weight) induced two hepatocellular tumors in 27 animals (7%). But the combination induced hepatocellular tumors in 3 of 27 (11%) rats at 42 hours and in 7 of 34 (21%) rats at 60 hours after the carbon tetrachloride was administered. Pound et al. (1973b) observed that the single dose of carbon tetrachloride induced a considerable increase in the synthesis of rat liver DNA, which reached its maximum 60 hours after treatment.

Other investigators have studied the effects of carbon tetrachloride on nitrosamine-induced liver tumors. Schmähl et al. (1965) fed NDMA and carbon tetrachloride concurrently to rats. A high incidence (75%) of liver tumors was obtained in animals receiving the nitrosamine with or without carbon tetrachloride, but tumor latency was somewhat reduced by the combined treatment. Taylor et al. (1974) reported a similar experiment in which aminopyrine and sodium nitrite led to a higher incidence of hepatocellular tumors in

0 of 15 did so in the absence of this chemical). The implication of these experiments with carbon tetrachloride is that the liver, normally having a very low rate of cell proliferation, is made more sensitive to carcinogenesis induced by N-nitroso compounds in the presence of agents that stimulate proliferation.

In a more recent study, carbon tetrachloride was found to enhance NDEA-induced hepatocarcinogenesis when it was administered before and/or after the N-nitroso compound (Pound and McGuire, 1978). A control group of the random-bred mice used in the study did not develop hepatocellular neoplasms during the 1-year course of the experiment. Furthermore, administration of carbon tetrachloride alone did not induce neoplasms. The investigators found that a single intraperitoneal injection of NDEA at 80 mg/kg body weight induced 26 hepatocellular tumors in 14 of 29 mice after 1 year. When seven doses of carbon tetrachloride were administered after NDEA, the number of tumors was nearly doubled (46 tumors in 21 of 26 mice). Administration of a single dose of carbon tetrachloride 24 hours prior to NDEA tripled the number of neoplasms (79 tumors induced in 20 of 26 mice). Finally, when carbon tetrachloride was administered before and after the NDEA, 172 tumors were induced in 28 of 28 mice -- a synergistic, sixfold enhancing effect. The results suggest that carbon tetrachloride enhances NDEA-induced hepatocarcinogenesis at both initiation and promotion stages of tumor development, probably by inducing hepatic parenchymal cell proliferation in response to cellular necrosis.

The carcinogenic effects of N-nitroso compounds can also be enhanced if administered with other carcinogens having a similar organotropy (Schmährl, 1980). In one study, during which equally carcinogenic doses of NDEA and 4-dimethylaminoazobenzene were fed to rats (Schmährl et al., 1963), the induction time for liver tumors greatly shortened when the carcinogens were fed together (153 days) in comparison to the time required when NDEA or 4-dimethylaminoazobenzene was given (233 and 235 days, respectively). In a later experiment, four liver carcinogens (NDMA, NDEA, NMOR, and p-dimethylaminoazobenzene) were administered in doses that did not cause liver cancer when they were applied separately, i.e., in subthreshold doses (Schmährl, 1970). The investigator speculated that if the effects were additive, some rats would develop carcinomas of the liver after 600 or 700 days. The results of the study supported the speculation, since 50% of the rats developed carcinomas by 700 days.

Short-Term, In Vivo Bioassays. As indicated earlier, most spontaneous neoplasms develop from initiated cells through a

organs including the skin and lungs of mice and the liver of rats, but none of them are considered to be adequate substitutes for lifetime studies. Recent studies of NDEA-induced hepatocarcinogenesis suggest that it might be useful to develop short-term tests for agents that are carcinogenic for the liver.

There is an increasing body of direct and indirect evidence that hepatocellular carcinomas in rats develop from enzyme-altered hyperplastic foci or islands (Goldfarb and Pugh, in press). Scherer and Emmelot (1976) induced ATPase-deficient hepatocellular foci by administering NDEA to adult rats that had been partially hepatectomized 24 hours earlier. They reported a progressive increase in the size of foci over time. Furthermore, they documented a linear response in the incidence of islands over a broad range of doses of the carcinogen. This observation suggested to the authors that islands arose from normal cells after a single carcinogen "hit" and that the further progression to trabecular carcinomas required additional "hits." In another study, Kunz et al. (1978) plotted the time of NDEA treatment against decreasing daily intake of the carcinogen on a log-log scale. They observed that the relationship for the induction of GGTase (γ -glutamyltranspeptidase) positive cells in 1% of the liver section area and for 50% mortality due to liver tumors (Druckrey, 1967) was described by straight lines with similar slopes. Thus, the relationship:

$$K = \text{daily dose of carcinogen} \times \text{time}^{2.3},$$

which was originally described by Druckrey (1967) for the induction of malignant neoplasms, also applies to the development of enzyme-altered islands. Of practical importance for devising a short-term in vivo bioassay was the observation that when island number, average area of islands, and total island areas were determined on tissue slides, the total island area was statistically most sensitive for quantifying the hepatocarcinogenic effect.

Recent studies in mice suggest that this species may also be useful in short-term bioassays for assessing the hepatocarcinogenic potency of N-nitroso compounds. Ribonucleic acid-rich hepatocellular foci with high nuclear-to-cytoplasmic ratios and a deficiency of glucose-6-phosphatase activity were found to increase in number and size following a single injection of NDEA in 15-day-old mice (Goldfarb et al., 1981). Since the foci were first noted 10 weeks following injection of the carcinogen (but 20% of them were found to invade terminal hepatic veins by 20 weeks) and since they acquired increasing

degrees of anaplasia during the course of the experiment, they were considered to be the precursors of trabecular hepatocellular carcinomas. The progressive, irreversible nature of the lesions was also supported by their almost spherical shape and high thymidine-labeling indices (10- to 80-fold times greater than "background" hepatocytes). However, NDEA is known to be a potent carcinogen. No studies of hepatocellular foci and less potent N-nitroso hepatocarcinogens have been reported.

Summary and Discussion: Carcinogenesis

Data on Humans. Perhaps the greatest limitation in the epidemiological studies described in this chapter is their lack of sufficient data on the history of exposure to nitrate, nitrite, and N-nitroso compounds for the individuals who develop cancer. One of the most thorough attempts to accumulate such data has been made by Correa and his colleagues (Correa et al., 1976, 1979; Cuello et al., 1976) in their studies of gastric cancer in Colombia. Despite the difficulties of ascertaining dietary intake and the inadequate medical diagnoses in poorly developed regions, these investigators were able to document the high frequency of both stomach cancer and dysplasia in certain areas of that country. It is likely that dysplasia is a true precursor, but this has not been proven. That nitrate, nitrite, or N-nitroso compounds play a role in the etiology of dysplasia and the subsequent development of stomach cancer in Colombia is plausible, but this, too, has not been proven. The finding of a negative association between consumption of vegetables containing the nitrosation inhibitor vitamin C lends further indirect support to this hypothesis.

Carefully conducted studies of populations in Chile, Japan, Iran, and China do not demonstrate a clear causal link between exposure to nitrate and nitrite and the risk of gastric and esophageal cancer in humans, nor do they rule out alternative causes. One study conducted in Chile showed an inverse correlation between stomach cancer and urinary nitrate levels. Studies of Japanese in Hawaii and in Japan were rigorous, but did not focus on N-nitroso compounds. In Iran, nitrate and nitrite intakes were similar in areas with a high and low incidence of esophageal cancer. The Chinese have recommended dietary changes to discourage the use of pickled vegetables and encourage the consumption of foods containing ascorbic acid. Further study is needed to determine if these recommended changes affect the incidence of esophageal cancer in that

in these two studies also suggests a possible causative role for nitrate, nitrite, and/or N-nitroso compounds.

Despite the valuable research opportunities, studies in certain occupational groups, such as rubber-industry workers, have not examined the exposure to nitrate, nitrite, or N-nitroso compounds in those who have developed cancer.

In summary, epidemiological studies have failed to provide convincing evidence that exposure is associated with cancer. Future studies to examine this association will require clearly defined populations, well-documented conditions of exposure for an adequate length of time, and documentation that the exposed individuals and not the controls are getting the cancers. In addition, alternative causations such as the presence of other, possibly carcinogenic substances in the environment must also be considered.

Data on Animals. The data on the carcinogenicity of nitrate and nitrite in animals are not definitive. However, there is convincing evidence that nitrite can react with nitrosatable agents in the acidic conditions of the stomach to produce N-nitroso compounds, most of which, when adequately tested in animals, have been proven to be carcinogenic. The impact of in vivo formation of N-nitroso compounds on cancer induction in humans cannot be determined with precision because the amounts of N-nitroso compounds formed following administration of nitrite and amines or amides at the low doses to which humans are normally exposed are not yet known. A further complication is the wide range (three orders of magnitude) in the carcinogenic potency of the various nitrosamines.

In estimating the extent to which humans are exposed as a result of such nitrosation reactions, it is important to use peak concentrations of nitrite and nitrosatable agents such as those that may occur after a meal, rather than the average daily intake, if meaningful results are to be obtained. It may also be helpful to focus on the importance of N-nitroso compound formation when higher levels of nitrosatable agents, such as certain drugs, are deliberately administered.

The original observations of Mirvish and his colleagues (1972b) that the formation of nitrosamines is inhibited by ascorbic acid have been confirmed, and a variety of nitrosation inhibitors (e.g., ascorbic acid) and accelerators (e.g., certain phenols) have been discovered. Again, there is a need for a more quantitative assessment of the availability of inhibitors and accelerators in the gastric contents of humans and animals, or in normal and specialized diets, to permit an adequate assessment of the hazards of in vivo nitrosation (see

Nitrosamides that do not require metabolic activation may be more potent carcinogens in rapidly proliferating fetal tissues than in adult tissues. For example, nitrosoureas have a strong propensity to induce tumors in the nervous systems of rats when administered transplacentally. Similarly, the activity of agents that increase cell proliferation in the liver can enhance carcinogenicity of N-nitroso compounds. The formation of carcinogenic N-nitroso compounds can also be enhanced or inhibited by the presence of certain chemical anions or reducing agents.

Despite the difficulties in extrapolating carcinogenicity data from animals to humans, data indicate that N-nitroso compounds will probably be found to be carcinogenic in humans. Two important facts support this conclusion: (1) These compounds are carcinogenic in every species tested thus far. (2) Humans are apparently capable of converting nitrosamines to the metabolic intermediates that alkylate the DNA. These intermediates are presumably the ultimate carcinogens (Montesano and Magee, 1970).

MUTAGENICITY {

Assays for mutagenicity and other forms of genotoxicity may be valuable as indicators of mutagenic potential of a chemical in vivo and predictors of whether a chemical may be a carcinogen. In this section, results of mutagenicity assays for nitrate, nitrite, and N-nitroso compounds are described and areas requiring further research are identified.

Nitrate and Nitrite

The only published information on the mutagenicity of nitrate appeared in an abstract prepared by Konetzka (1974), who studied the mutagenicity in Escherichia coli under aerobic and anaerobic conditions. He observed a hundredfold increase in mutant colonies in the presence of nitrate under anaerobic conditions, but no increase under aerobic conditions. In contrast, Salmonella typhimurium was not susceptible to mutagenesis by nitrate, even under anaerobic conditions. The author postulated that the observed mutations were due to the reduction of nitrate to nitrite -- not directly to the nitrate itself.

Nitrite, as nitrous acid, may lead to mutations by one of three mechanisms (Zimmermann, 1977):

Gleier, 1958; Schuster and Senanayake, 1958; Tessman, 1959). However, spontaneous deaminations are frequent, even in the absence of nitrous acid, and DNA repair systems that correct such lesions are present in bacteria and probably in all healthy mammalian cells (Hartman, 1980; Lindahl, 1979).

- In organisms with double-stranded DNA, mutagenesis by nitrous acid may also proceed by other mechanisms (Frankel et al., 1980; Hayakawa et al., 1978; Kotaka and Baldwin, 1964; Litman, 1961; Oeda et al., 1978). It may proceed the creation of intra- or interstrand cross-links between purine residues, which lead to helix distortion (Dubelman and Shapiro, 1977). The induction of helix-distorting lesions by nitrous acid appears to be enhanced by the presence of molecules proximate to DNA, such as polyamines, glycols, alcohols, or phenols (Murphey-Corb et al., 1980; Thomas et al., 1979b).

- In a third mechanism, nitrite reacts with nitrosatable substrates to produce N-nitroso compounds that are known carcinogens and mutagens. It may also contribute to the formation of C-nitroso, aryl-nitroso, and S-nitroso compounds, some of which are carcinogenic and mutagenic (Gilbert et al., 1980; Natake et al., 1979). C-Nitroso compounds such as nitrosophenols are generally regarded as nongenotoxic. Specific aryl-nitroso compounds are carcinogenic, but they result from the metabolism of aromatic amines rather than from direct nitrosation. S-Nitroso compounds have not been investigated because of their instability.

The few in vivo studies that have been conducted have provided little useful information on the mutagenicity of nitrite. In a host-mediated assay, nitrite was without activity (Couch and Friedman, 1975); however, the nitrite may never have reached the site where the test organisms were located. In vitro assays of cultured mouse cells demonstrated nitrite-induced mutagenicity only when substrate concentrations higher than 1 mM were obtained (Kodama et al., 1976).

Despite the unequivocal demonstration of nitrite-induced mutagenicity in in vitro systems in which single-stranded and double-stranded DNA are the targets, no evidence that this occurs in intact mammalian organisms has been provided either directly or by host-mediated assays.

N-Nitroso Compounds

Many N-nitroso compounds are shown to be mutagenic when assayed under certain conditions. Bacterial mutagenicity assays of nitrosamines require supplementation with animal-derived enzymes to detect mutageni-

compounds have been shown to be mutagenic in microbial systems (McCann et al., 1975; Montesano and Bartsch, 1976).

There have been several reviews of the data on the mutagenicity of N-nitroso compounds (Montesano and Bartsch, 1976). The Ames Salmonella typhimurium microsome test (Ames, 1979; Ames et al., 1973a,b) is the system applied most often to these compounds. The original Ames method was not very responsive to N-nitroso compounds, but by using a different technical approach, i.e., preincubating the nitrosamine in liquid suspension with the bacteria, investigators were able to obtain highly satisfactory results (Yahagi et al., 1977). Many such tests have been performed using Salmonella tester strains TA1535, TA1537, TA1538, TA98, and TA100. Mutations are produced in one or more of these strains, depending on the nature of the test chemical (Andrews et al., 1980). S. typhimurium is by no means the only monocellular organism to be used in this way. Other microbes, such as E. coli, and yeasts, such as Saccharomyces cerevisiae, are also suitable (Elespuru and Lijinsky, 1976; Larimer et al., 1978). Test systems have been reviewed by Hollstein et al. (1979) and Brusick (1980).

Microbial tests have also been used to demonstrate the nitrosation of amines by nitrite (Andrews et al., 1980; Couch and Friedman, 1975) and the ability of ascorbate to inhibit the mutagenicity of nitrosamines (Guttenplan, 1978).

Many investigators have used the microbial mutation assay to examine food as well as fluids or excreta from the human body for traces of mutagens (Bruce et al., 1977; Lin et al., 1979; Scheutwinkel-Reich et al., 1980). Studies of this nature need very careful evaluation for two reasons: artifacts may form during mutagen extraction (Iwaoka et al., 1981) and mutagenicity may be due to agents other than N-nitroso compounds.

N-Nitroso compounds have been used in numerous other test systems as well (Hollstein et al., 1979; Montesano and Bartsch, 1976; Neale, 1976). Drosophila melanogaster was the first organism shown to develop germ-line mutations as a result of treatment with N-nitroso compounds (Coulston and Olajos, 1980; Rapoport, 1948; Vogel and Sobels, 1976). Russell et al. (1979, 1980) reported that nitroso-ethylurea is a potent mutagen for germ-line mutations in the mammal, in contrast to nitrosomethylurea, which is only weakly mutagenic for mammalian germ cells (Ehling et al., 1968). Taken together, these results provide substantial evidence of the mutagenic potential of the N-nitroso compounds; however, for a class of compounds containing mostly established carcinogens, the qualitative prediction for car-

provide results that correspond to carcinogenic potency in varying degrees. Langenbach et al. (1980) determined the mutagenic potency of four compounds known to be carcinogenic in the hamster pancreas. The agents, in descending order of carcinogenic potency, were nitrosomethyl-2-oxopropylamine (MOP), nitrosobis(2-oxopropyl)amine (BOP), nitroso-2-oxopropyl-2-hydroxypropylamine (HPOP), and nitrosobis(2-hydroxypropyl)amine (BHP). These investigators reported that the use of liver S-9 fraction placed the four carcinogens in an order of mutagenic potency in the Ames test with S. typhimurium TA1535 that was almost the inverse of carcinogenic potency (Figure 9-1). However, when hamster lung V79 cells were used and resistance to ouabain was the method of selecting mutants, cocultivation of uninduced primary explants of hamster liver cells with the mutable V79 cells placed the four nitroso compounds in the correct order of potency.

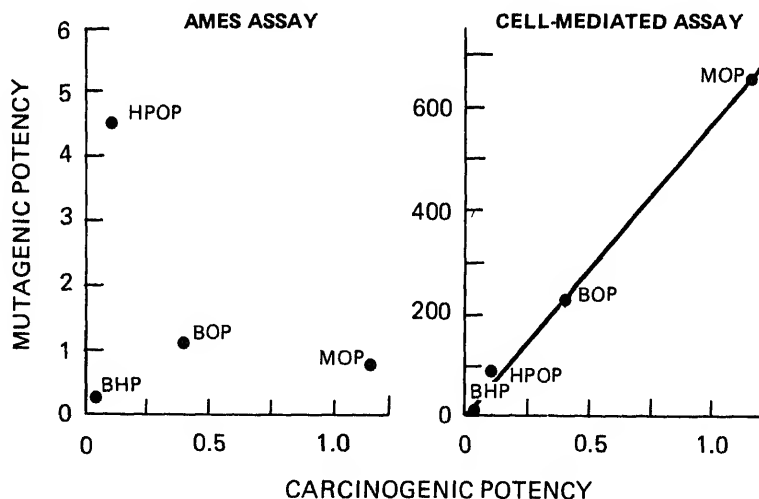


FIGURE 9-1. Relationship of carcinogenicity and mutagenicity in the Ames Salmonella and cell-mediated assays. Carcinogenic potency is expressed as the reciprocal of the lowest weekly dose that produced a tumor incidence of at least 60% to 70%. From Langenbach et al., 1980.

convincing results than the microbial test probably lies in the use of whole liver cells that are better able to mimic metabolic activation in vivo than the crude S-9 fraction. Jones et al. (in press) have now examined 27 N-nitroso compounds by the hepatocyte-mediated V79 mammalian cell method and obtained a high degree of correlation between carcinogenic and mutagenic potency. In their study, they defined mutagenic potency as the concentration of nitrosamine that yields a frequency of mutation 10 times higher than the frequency of spontaneous mutation. The index for carcinogenicity was defined as a function of the nitrosamine dose and time to death resulting from tumors in 50% of the exposed animals. Using these indices, the authors established a linear relationship between the degrees of carcinogenicity and the degree of mutagenicity for the nitrosamines with a p-value of 0.0001.

In contrast, the correlation of mutagenic potencies for N-nitroso compounds tested in the bacterial systems does not correlate very strongly with potency determinations from carcinogenicity studies (Ames, 1979; Meselson and Russell, 1977). However, for certain N-nitroso compounds with closely related structures (Andrews et al., 1980; Preussmann et al., 1979; Rao et al., 1977, 1979a,b; Wakabayashi et al., 1981), which are only minimally affected by use of the S-9 fraction for metabolic activation, some rough correlations have been obtained.

Variation in Mutagenicity Data. Variation in the results from assays stems from three major considerations. First, potentially mutagenic events take place at multiple sites on DNA (Kröger and Singer, 1979; Singer, 1979). The position of these events (i.e., alkylation of DNA) and, thus, the resulting mutagenic effectiveness of different N-nitroso compounds depend on the nature of the alkylation (McMahon et al., 1979). For example, nitrosomethylurea is more potent than nitrosoethylurea in the Ames test (Lee et al., 1977), in the host-mediated assay (Couch and Friedman, 1975), in cytotoxicity tests, and in tests for HGPRT (hypoxanthine guanine phosphoribosyl-transferase) locus mutations in Chinese hamster ovary (CHO) cells (Couch and Hsie, 1978). In contrast, E. coli strains carrying the trpA58 mutation (Hince and Neale, 1974) and strain WP-2 derivatives (Garner et al., 1979) exhibit a greater mutagenic responsiveness to nitrosoethylurea than to nitrosomethylurea. Lawley (1980) reported that the ratio of O⁴-thymine and O⁶-guanine alkylation is approximately 0.05 for nitrosomethylurea and 0.3 for nitrosoethylurea. This suggests that nitrosomethylurea would favor G/C to A/T mutations, whereas nitrosoethylurea would favor A/T to G/C mutations. Thus, nitrosomethylurea would be more active in the Ames tester bacteria that carry the histidine G46 missense mutational triplet

shift. Similarly, the trpA58 mutation (GAC) could form true wild-type revertants only by reversion to GGC -- an A/T to G/C base-pair change (Nicholas and Yanofsky, 1979; Yanofsky, 1967).

Differences in the results of mutagenicity assays may also be due to a nonlinear relationship between dose and mutagenic response, which may be a consequence of the necessity for metabolic activation (Guttenplan, 1979; Guttenplan et al., 1976; Malling, 1971; McCann et al., 1975). However, even for the direct-acting nitrosoureas, the dose-response curve at the lowest doses shows an area of minimum response, followed at increasing doses by a strong and increasing response (Brundrett et al., 1979; Franza et al., 1980; Guttenplan, 1979; Lee et al., 1977). The slope of the biphasic dose-response curve is dependent on alkyl chain length and the test protocol (Brundrett et al., 1979). Other direct-acting alkylating mutagens, i.e., linear aryl-monoalkyl triazines, show similar biphasic dose-response curves (Endo et al., 1980; Thomas et al., 1979a).

The biphasic nature of the dose-response curve for the mutagenic response to a dose of alkylating agent may be partly explained by an adaptive response in repair proficiency in the repair of O⁶-guanine lesions at low levels of the alkylating agent and the saturation or inactivation of this system at higher levels (Cairns, 1980; Jeggo et al., 1977; Karran et al., 1979; Robins and Cairns, 1979; Samson and Cairns, 1977). Similar attributes have been demonstrated for DNA repair at O⁶-guanine in vivo in mammals (Buckley et al., 1979; Montesano et al., 1979; Pegg, 1978).

Finally, differences in results from mutational testing may be due to the lack of standardization among the many laboratories conducting these studies.

Correlation Between In Vitro and In Vivo Mutagenicity Assays.
In vitro mutagenicity tests are valuable because they can be applied to many more chemicals than can in vivo tests for mutagenicity or carcinogenicity, which are limited by their requirement for large numbers of animals.

Unfortunately, there is little information on chemically induced mutation in whole animals for comparison with the results of in vitro tests. However, it is clear that the distribution of the chemical in the whole animal and its pharmacodynamic fate provide one source of difference because such considerations do not arise in in vitro tests. Furthermore, there is relatively little knowledge about whether the germ cells or embryo are protected from the effects of electrophiles, such as nucleophile-containing molecules. Finally, it is not known

forms. Thus, predictions of mutagenicity in whole animals based on in vitro results must be regarded with reservations. This is especially true for nitrosamines that require metabolic activation than for nitrosamides that do not, primarily because, unlike the normal intact liver, the S-9 fraction (prepared from livers of rats treated with phenobarbital or Aroclor) may not activate or detoxify carcinogens (Clayson, 1980; Wright, 1980).

Another major difference between in vitro assays and in vivo tests can be observed in DNA repair mechanisms. Chemically induced lesions in DNA are often removed by one or more DNA repair systems. Tester cells in both microbial and mammalian cell mutation assays are usually more sensitive to chemical effects when their DNA repair capability is deficient, which leads to another area of discordance between in vitro and in vivo tests. There would still be discrepancies even if cells with a normal DNA repair capacity were used, since the repair capacity of cells from humans is markedly different from that of cells from rodents. Finally, the tumor progenitor cell in which the test chemical has induced one or more genetic errors must be developed within the constraints of the body systems to a frank clinical tumor; no such constraints affect the in vitro system. Despite these limitations, however, most N-nitroso compounds that are carcinogenic in animals are mutagenic when tested under appropriate conditions, although exceptions do occur (Rao et al., 1979).

Summary and Discussion: Mutagenesis

Nitrate does not appear to be directly mutagenic. In microbial systems, nitrite may be mutagenic by three different mechanisms: deamination of DNA bases in single-stranded DNA; formation of intra-strand or interstrand lesions leading to helix distortions in double-stranded DNA; and formation of mutagenic N-nitroso compounds. In mammalian systems, however, there is no evidence that nitrite is mutagenic (except for one study, in which a high dose of nitrite was used).

Despite several limitations of the in vitro mutational assays stemming from differences in metabolic activation and DNA repair mechanisms among species, appropriate mutagenicity tests on N-nitroso compounds have usually provided a high level of correlation with whole animal studies, although exceptions do occur. The majority of N-nitroso compounds considered for testing are usually positive for carcinogenicity and, under appropriate conditions, for mutagenicity. This leads to a good degree of correlation at the qualitative level, and quantitative correlations have been observed in mammalian muta-

Exposure to high doses of nitrate, nitrite, and N-nitroso compounds has been associated with a variety of adverse health effects in humans and other animal species. This section contains a review of data pertaining to the role of these compounds in the induction of disease in humans and descriptions of those diseases. Data on diseases in animals associated with exposure to nitrate and nitrite are also reviewed along with data from experiments on toxicity and teratogenicity of N-nitroso compounds in animals.

Toxic Effects in Humans: Nitrate and Nitrite

The toxic effects of nitrate and nitrite have been extensively reviewed (Archer, in press; Corr  and Breimer, 1979; Green et al., in press; National Academy of Sciences, 1978; World Health Organization, 1978). The committee has summarized this information and described the most prevalent toxic effects. Nitrate and nitrite are discussed together since most toxic reactions are due to nitrite derived from bacterial reduction of nitrate, either prior to ingestion or within the host (Chapter 8).

Corr  and Breimer (1979) and Burden (1961) have summarized the literature documenting the toxic and lethal levels of nitrate and nitrite. Different studies have reported that the lethal level of nitrate for a 60-kg adult ranges from 4 to 50 g, whereas for nitrite, it ranges from 1.6 to 9.5 g (Corr  and Breimer, 1979). Although the criteria for toxicity vary, most authors accept as a criterion for toxicity a single dose that will induce methemoglobinemia. In four studies, the listed toxic dose for nitrate ranged between 2 and 4 g, whereas for nitrite it ranged from 60 to 500 mg (Corr  and Breimer, 1979).

Methemoglobinemia. Methemoglobinemia is the most prevalent and potentially the most serious known complication of nontherapeutic, excessive intake of nitrate and nitrite. The condition, characterized clinically by cyanosis and anoxia, is due to defective transport of oxygen by high levels of circulating methemoglobin. Methemoglobin is an oxidation product of hemoglobin in which the ferrous iron of hemoglobin is oxidized to the ferric form (Jaff , 1981). The mechanisms by which nitrite causes methemoglobinemia are complex, apparently involving the formation of intermediate complexes between hemoglobin and nitrite redox products (Kiese, 1974).

After formation of methemoglobin, oxygen can no longer reversibly bind to red blood cells. If the oxidation damage proceeds far enough, the hemoglobin may be irreversibly damaged, leading to the formation of hemochromes. Finally, the hemoglobin may be denatured and pre-

only from an inability of the oxidized hemoglobin to transport oxygen, but also from interference by methemoglobin with normal delivery of oxygen transported by oxyhemoglobin. The latter effect was originally suggested by Darling and Roughton (1942) and has been confirmed in more recent years (Brewer, 1972; Enoki et al., 1969). Approximately 1% of the hemoglobin circulates as methemoglobin in the normal adult; in children, it is usually less than 2%. Clinical cyanosis usually appears when approximately 10% of the hemoglobin is converted to methemoglobin; symptoms of cerebral anoxia supervene at approximately 20%; and stupor, coma, and death usually result when conversion levels reach 60% or more.

Patients with mild methemoglobinemia may be treated with oral doses of ascorbic acid administered 3 times daily. When patients are in extremis, immediate intravenous injection of methylene blue, which rapidly reverses the methemoglobinemia, is usually life-saving.

There are no proven cases of chronic sequelae of nitrite- or nitrate-induced methemoglobinemia in humans. Petukhov et al. (1972) reported slightly delayed reaction time in children drinking water containing high levels of nitrate (average, 105 mg/liter); however, this effect, if confirmed, may not be specifically related to methemoglobinemia. A delayed reaction time was also observed in mice that were given drinking water containing up to 2,000 mg of sodium nitrite per liter and enough ascorbic acid to inhibit the formation of methemoglobin (Shuval and Gruener, 1972).

The true incidence of methemoglobinemia in the United States is not known since there are no regulations requiring that cases be reported. However, approximately 2,000 cases were documented in North America and Europe between 1945 and 1971 (Shuval and Gruener, 1971). In the United States, from 1939 to 1950, there were reports of approximately 320 cases of methemoglobinemia in infants who ingested nitrate-rich well water (Walton, 1951). In the Federal Republic of Germany, where some of the best data are available (Simon et al., 1964), 745 cases between 1956 and 1964 were attributed to water containing high concentrations of nitrate. Private wells had supplied the water for 97.3% of these cases; public water supplies had been used by the remaining 2.7%. Eighty-four percent were related to water supplies containing more than 100 mg of nitrate per liter. Nitrite was detectable in samples of water used by only 10% of these cases.

Infants are at greatest risk of developing methemoglobinemia from excessive intake of nitrate. This increased susceptibility is related to at least four factors (Anonymous, 1966; Lee, 1970;

reductase or its cofactor NADH (reduced nicotinamide adenine dinucleotide), which are necessary to maintain iron in its reduced state (Ross and Desforges, 1959). Third, on a weight basis, infants consume approximately 10 times more water (the most important source of nitrate in the etiology of methemoglobinemia) than do adults (Burden, 1961). Finally, relative achlorhydria in the very young probably favors overgrowth of nitrate-reducing organisms in the upper gastrointestinal tract (Walton, 1951; see also Chapter 8). Ewing and Mayon-White (1951) reported that only infants with a gastric pH above 4.0 developed methemoglobinemia after ingesting water containing high levels of nitrate. Similarly, infantile epidemic gastroenteritis may favor the development of methemoglobinemia when the upper gastrointestinal tract becomes overgrown with bacteria (Fandre et al., 1962).

Several other categories of individuals with altered physiological states or with either hereditary or acquired disease may also be predisposed to the development of nitrite- or nitrate-induced methemoglobinemia. These include pregnant women (Metcalf, 1961), individuals with glucose-6-phosphate dehydrogenase deficiency (Kohl, 1973), adults with reduced gastric acidity (including those being treated for peptic ulcer or individuals with chronic gastritis or pernicious anemia), and a rare group with a hereditary lack of NADH or methemoglobin reductase activity in their red blood cells (Scott, 1960). This hereditary enzyme deficiency seems also to account for the somewhat increased incidence of methemoglobinemia among Alaskan Eskimos and Indians (Scott and Griffith, 1959; Scott and Hoskins, 1958). Individuals with hereditary structural abnormalities in hemoglobin, referred to as hemoglobin Ms, are probably also at increased risk from dietary nitrate or nitrite. In these unusual hemoglobinopathies (two types have been described), substituted amino acids in the globin moiety create new, very strong bonds with the heme iron, maintaining it in the ferric state (Jaffé and Heller, 1964).

Nitrate in drinking water is the factor most commonly associated with the genesis of methemoglobinemia. In the most comprehensive study of its kind, the cases of water-induced methemoglobinemia were classified according to the concentration of nitrate-nitrogen in water used in the preparation of infant formulae (Walton, 1951). Although there were shortcomings in both clinical diagnosis and water analysis, a very obvious finding was the absence of methemoglobinemia in populations whose drinking water contained nitrate-nitrogen concentrations less than 10 mg/liter. In addition, only 2.3% of the cases were infants who drank water containing 10 to 20 mg of nitrate-nitrogen per liter. These figures seem to validate

of nitrite has been implicated in all of the other, exceedingly rare cases of secondary methemoglobinemia. An unusual case of methemoglobinemia was traced to the use of nitrate-rich water in a home dialysis unit (Carlson and Shapiro, 1970). Vegetables containing high levels of nitrate, such as spinach, pose a potential threat when their processing or refrigeration is delayed or inadequate. However, less than two dozen cases of methemoglobinemia associated with spinach have been documented, and most of these occurred in the Federal Republic of Germany (Anonymous, 1966) where it has been customary to prepare pureed vegetables by retaining the nitrate-enriched water (Sinios and Wodsak, 1965). For each case of infantile methemoglobinemia traced to this source, toxic levels of nitrite were held responsible. The conversion of nitrate to nitrite in spinach is probably due to the activation of nitrate reductase, which is present in high concentrations in the vegetable (Paneque et al., 1965) (see Chapter 5) or to a similar enzymatic action by bacterial contamination (see Chapter 8).

Ingestion of improperly processed sausage (Bakshi et al., 1967; Orgeron et al., 1957) or fish (Singley, 1962) has also been associated with the development of methemoglobinemia. In one outbreak, sodium nitrite was inadvertently substituted for table salt in a restaurant (Greenberg et al., 1945). In all cases attributed to food processing, the levels of sodium nitrite in the food were far in excess of the permitted maximum residual of 200 mg/kg (U.S. Department of Agriculture, 1970).

Miscellaneous Effects. Excess intake of nitrate esters, which are vasodilators used to treat angina pectoris, may induce headache, facial flushing, and, in severe cases, even syncope and hypotension (Opie, 1980). A remarkable consequence of chronic exposure to relatively high levels of nitrate esters is the development of tolerance to the vascular effect. This has been observed in workers in explosives factories who frequently suffer from severe headache, dizziness, and postural weakness during the first few days of employment in factories manufacturing nitroglycerin. These workers soon develop tolerance to the compound. To maintain this short-lived tolerance while away from work, the workers have learned to rub the nitrate esters into their skin, thereby preventing recurrence of symptoms upon their return to the workplace.

A potentially serious effect of chronic exposure to nitrate esters is the now well-documented development of dependence on organic nitrate derivatives (Nickerson, 1975). This dependence was first observed in some explosives workers who were free of clinical atherosclerosis; however, after several days away from work, they developed severe myocardial ischemia and even myocardial infarction. Although

The literature contains only a few reports of fatalities resulting from exposure to nitrosamines. In two of the earliest reports, occupational exposure to NDMA resulted in acute liver necrosis, which later developed into cirrhosis. In one case, death resulted from the exposure (Barnes and Magee, 1954; Freund, 1937). In a more recent report, criminal poisoning was proven (Fussgaenger and Ditschuneit, 1980). The victim had apparently been fed repeated doses of NDMA over many months and ultimately died of hepatic decompensation and cirrhosis. An autopsy of the liver showed fibrosis and hyperplastic nodules (cirrhosis). In another incident where NDMA poisoning was suspected as a cause of death, the liver from the victim was subjected to DNA analysis (Herron and Shank, 1980). Very high levels of methylated DNA adducts (O⁶-methylguanine and 7-methylguanine) known to result from exposure to NDMA were found in the hepatic DNA from this person, but were absent from the liver and kidneys of the patients who had died from other causes. Based on the known rate of in vitro metabolism of nitrosamines by the human liver, the authors speculated that the victim had been exposed to 20 mg or more of NDMA per kilogram of body weight.

Toxic Effects in Other Species: Nitrate and Nitrite

Turner and Kienholz (1972) and Emerick (1974) have reviewed nitrate-nitrite toxicity in livestock and other animals. Although the toxic effect of most agents is similar in various species, including humans, there appear to be large differences in the effective dose of the agent required to produce these effects among species. In general, ruminants are much more susceptible to the toxic effect of nitrate than are nonruminants, probably because of the longer retention and greater opportunity for bacterial reduction in the rumen. For example, the lethal dose of nitrate in pigs is 300 mg/kg body weight, a level that is approximately 4 times greater than the lethal dose in ruminants (Gwatkin and Plummer, 1946).

Tolerance to nitrate has been induced experimentally in animals. In one extreme example, lambs weighing 32 kg were adapted to increasing levels of potassium nitrate and were able to consume rations containing 1.28% of the compound without showing any obvious ill effects (Sokolowski et al., 1960).

Rats were found to tolerate 10,000 mg/kg sodium nitrate in the diet for 2 years with no adverse reaction, and 50,000 mg/kg in the diet of sodium nitrate produced only mild retardation of growth. Dogs were not adversely affected when they were fed a diet containing 20,000 mg/kg sodium nitrate for 105-125 days.

As expected, orally administered sodium nitrite was 10 times more toxic in ruminants than in nonruminants (Emerick, 1974). Intravenous injections of approximately 6 mg of nitrite-nitrogen per kilogram of body weight have produced consistent moderate to severe methemoglobinemia in pigs (Emerick et al., 1965), dogs (Jensen and Anderson, 1941), and ruminants (Emerick et al., 1965). Some reports indicate that rats are relatively resistant to the long-term toxic effects of nitrite. The feeding of sodium nitrite to two generations of rats at 240 to 460 mg/kg in the diet was without effect on litter size, infant mortality, growth rate, or longevity (Shank and Newberne, 1976). However, 2,000 to 3,000 mg of sodium nitrite per liter of drinking water given to rats for 2 years induced heart and lung damage. After exposure for only 2 weeks to levels between 100 and 200 mg of sodium nitrite per liter of drinking water, rats had abnormal electroencephalogram patterns, which persisted after discontinuation of the treatment (Shuval and Gruener, 1972).

The effect of nitrate and nitrite on the reproductive capacity of farm animals continues to be of concern, especially since Thorp (1938) suggested that there was an increased incidence of abortion after animals ingested hay containing high levels of nitrate. However, more recent studies indicate that nitrate exerts no significant abortifacient effect on heifers and ewes at levels approaching those that induce fatal methemoglobinemia (Davison et al., 1964, 1965; Simon et al., 1958).

Emerick (1974) reported that chronic nitrate and nitrite intoxication induces a deficiency of vitamin A in a number of animals, including poultry, pigs, turkeys, and sheep. Most of the evidence indicates that nitrite, but not nitrate, depletes vitamin A in nonruminants by destroying it in the gut lumen under acidic conditions (Roberts and Sell, 1963; Sell and Roberts, 1963). In view of the generally low level of nitrate in feeds, grains, and water, Emerick (1974) believes that the effect of nitrate and nitrite on vitamin A utilization or storage is of no great significance, at least in poultry and swine.

activation. Other studies indicate that the hepatotoxicity of nitrosamines follows a marked impairment of protein synthesis. This is probably a consequence of defective RNA processing (Emmelot, 1964; Mager et al., 1965; Mizrahi and de Vries, 1965), which is believed to be responsible for a secondary defect in peptide chain initiation (Pegg, 1977). DNA synthesis is also acutely impaired after administration of NDMA, possibly because of decreased DNA polymerase activity (Salisbury and O'Connor, 1976) or the induction of lesions in the DNA template. In the rat, single toxic doses of NDMA (20 mg/kg body weight or greater) and NDEA (200 mg/kg body weight) result in striking central zonal necrosis of the liver (Barnes and Magee, 1954; Solt et al., 1977), which, for NDMA, begins within 6 hours. Milder hepatotoxicity, also accompanied by central zonal localization, resulted from administration of the heterocyclic nitrosamine NPYR (Hendy and Grasso, 1977).

Studies of nitrosamine-induced acute toxicity in organs other than the liver are relatively uncommon. Renal toxicity is of particular interest since it appears to correlate with the late appearance of neoplasia of the kidney in a manner somewhat similar to that in the liver. Administration of single, very high doses of NDMA to young rats resulted in a decrease in DNA synthesis in the kidney after only 24 hours. Focal periglomerular collections of replicating mesenchymal cells, which were observed on the second day, continued to enlarge, ultimately resulting in mesenchymal neoplasms. Since the replicating cells were located in precisely the same place as the toxically injured cells and since the degree of toxic injury and the tumor incidence were both dose dependent, these findings indicate a relationship between acute cellular damage and ultimate tumor development (Hard and Butler, 1970, 1971).

Greenblatt and Rijhsinghani (1969) reported that susceptibility for necrosis of the olfactory epithelium 48 hours after administration of a nitrosamine seemed to correlate with carcinogenic response in Syrian golden hamsters. The acute toxicity of nitrosoalkylamines generally decreased with increasing chain length, and at the LD₅₀ level, NDEA was a far more effective nasal toxin and carcinogen than was NDMA. The LD₅₀'s of many of these agents have been determined. The most potent is considered to be nitrosomethylbenzylamine, which has an LD₅₀ of 18 mg/kg body weight in rats (Druckrey et al., 1969; Shank, 1975). When measuring the LD₅₀'s of several carcinogens, such as NPiP, that are not carcinogenic in the liver, Mirvish (personal communication, 1981) observed that death occurs almost immediately after ingestion because of toxic effects on the nervous system characterized by convulsions.

to reflect susceptibility to the carcinogen. During the first week following a single injection of NDMA, the tritiated-thymidine-labeling indices of Type 2 pneumocytes, the putative precursor cell population of lung adenomas, were 2 times higher in GRS/A mice than in C3H/A mice. In contrast, labeling of hepatocytes was 2 times higher in C3H/A mice than in the livers of GRS/A mice during that same period. Since GRS/A mice develop lung adenomas, but C3H/A mice develop hepatocellular carcinomas, the early replicative difference may provide a measure of the organ's susceptibility to the carcinogen.

Teratogenic Effects. When administered to rats, Syrian golden hamsters, or minipigs during the first half of pregnancy, high doses of nitrosomethylurea or nitrosoethylurea induced brain and bone malformations (Ivankovic, 1979). Apparently, the route of administration was not important and high molecular weight homologues of the agents were less teratogenic than those with a lower molecular weight. A teratogenic dose-response effect was observed in BD rats that had been treated with single intravenous injections of nitrosoethylurea. The median teratogenic dose was 46 mg/kg body weight, corresponding to about one-fifth of the LD₅₀. If a linear extrapolation is valid, these limited data suggest that the maximum dose of nitrosoethylurea producing no teratogenic effect is approximately 20 mg/kg body weight. Administration of ethylurea with nitrite to rats during the first trimester of pregnancy resulted in in vivo formation of nitrosoethylurea, resulting in hydrocephalus in the offspring. However, when administered to the rats during the second half of pregnancy, the progeny developed neurogenic tumors. In a related study, Ivankovic et al. (1973) found that coadministration of ascorbate with the nitrosating mixture effectively blocked the induction of hydrocephalus.

Druckrey (1973) conducted a systematic investigation of the transplacental teratogenic and carcinogenic effects of a number of N-nitroso compounds in rats. He observed that chemical structure, manner of metabolic activation, and stage of fetal development all had a similar effect on teratogenesis and carcinogenesis. Malformations of the central and peripheral nervous system were evident in many instances and, similar to the studies by Ivankovic and his colleagues (1973), the administration of the agents early in embryogenesis caused brain malformations in the absence of neoplasms, whereas treatment with these compounds at later stages of pregnancy was associated with the development of a variety of neoplasms. Clearly, then, the site of action for the teratogenic effects is different from that of the carcinogenic effect.

Summary: Effects Other Than Carcinogenicity or Mutagenicity

Nitrate- and nitrite-induced methemoglobinemia in humans occurs mainly in infants and other predisposed individuals following ingestion of high levels of nitrate in drinking water or nitrite in prepared vegetables. The disease is characterized by cyanosis and anoxia, and is largely reversible by administering ascorbic acid or methylene blue, depending on the severity of the disease. N-Nitroso compounds can be toxic at very high levels of exposure, and death has resulted from irreversible liver damage in several instances.

In animals, nitrate and nitrite can be toxic, especially in ruminants. N-Nitroso compounds may cause fetal death and/or teratogenesis in several species that have been tested. This preliminary evidence of teratogenicity of N-nitroso compounds merits further research, especially because N-nitroso compounds are also mutagenic and carcinogenic in other test systems. The finding that nitrosamides are teratogenic is consistent with the findings that these compounds cause tumors transplacentally and that they are directly mutagenic (i.e., they do not require metabolic activation). It may be that nitrosamines are less active than nitrosamides transplacentally because they require metabolic activation and the necessary enzymes may not be produced in sufficient quantities in the developing fetus.

OVERALL SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

An excessive intake of nitrate or nitrite leads to the development of methemoglobinemia. The effect has been well-documented in humans, and a similar effect has been observed in animals exposed to high doses of these two chemicals. The disease appears to be induced by a complex mechanism in which nitrite oxidizes hemoglobin to methemoglobin -- a type of hemoglobin that is incapable of reversibly binding oxygen.

Evidence implicating nitrate, nitrite, and N-nitroso compounds in the development of cancer in humans is circumstantial. Several epidemiological studies of certain geographical/nationality groups have provided data that are consistent with the hypothesis that exposure of humans to high levels of nitrate and nitrite may be associated with an increased incidence of cancers of the stomach and esophagus. However, in none of these studies was there a direct attempt to investigate actual exposures of nitrate, nitrite, or N-nitroso compounds in individuals who develop cancer. In most of

or nitrite is carcinogenic. In animals, nitrate has not been shown to be directly carcinogenic or mutagenic. The limited data on nitrite indicate that it may not act directly as a carcinogen but that it is mutagenic in microbial systems.

In contrast, the N-nitroso compounds are clearly carcinogenic in every species of animals in which they have been tested. Positive results have been obtained for approximately 90% of the 300 N-nitroso compounds tested for carcinogenicity in one or more species. Most of these compounds are also mutagenic. Several nitrosamides have been shown to be teratogenic in animals. Thus, tests in animals provide strong evidence that N-nitroso compounds are likely to be carcinogenic in humans. These tests have also revealed the importance of enhancers and inhibitors of carcinogenicity. For example, agents that promote cell proliferation in the liver enhance carcinogenicity. In contrast, ascorbic acid, α -tocopherol, and other antioxidants can inhibit carcinogenicity by blocking the formation of N-nitroso compounds from the reaction of nitrite and nitrosatable substrates. These findings indicate that humans exposed to a chemical or virus that stimulates proliferation of liver cells may be predisposed to carcinogenesis induced by N-nitroso compounds. Moreover, they suggest that diets low in vitamin C content may create conditions favorable for the in vivo formation of N-nitroso compounds in humans.

The following recommendations of the committee are based on the data reviewed in this chapter.

1. The committee recommends that future epidemiological studies focus on correlating actual exposures to nitrate, nitrite, and N-nitroso compounds to incidence of cancer. Also, emphasis should be placed on determining other factors, such as the presence of precursor lesions affecting susceptibility. Where possible, exposure should be correlated with actual levels of nitrate, nitrite, and N-nitroso compounds in biological fluids such as blood, saliva, gastric juice, and urine.

2. Results of limited experiments suggest that nitrate is not carcinogenic or mutagenic. The committee recommends that future tests of the carcinogenicity of nitrate be correlated with the test species' ability to reduce nitrate to nitrite in the saliva, which may be of an important mechanism in humans.

3. Current evidence indicates that nitrite may not act directly as a carcinogen in animals. However, because of its mutagenicity in microbial systems and its possible role in the induction of stomach and esophageal cancer in humans, further testing in animals may be

carcinogen, a cocarcinogen, or a promoter.

4. Many N-nitroso compounds are clearly carcinogenic in laboratory animals and mutagenic in microbial and mammalian test systems; some are teratogenic in hamsters and rats. However, the value of these tests in the prediction of risk to humans is unknown. The committee recommends that future carcinogenicity assays emphasize quantitative assessment of potency and dose-response relationships as well as the qualitative outcome. It also recommends continued development of mammalian cell mutation assays with emphasis on the use of whole cells of liver and other tissues to provide a better model for the metabolism of the test agent in vivo. These tests should be extended to the use of human cells to learn more about the potency of the N-nitroso compounds in humans. Metabolic studies with human tissues may also help in this regard.

5. Premalignant lesions induced by N-nitroso compounds in humans and laboratory animals should be characterized and short-term in vivo bioassays should be developed to determine the carcinogenicity of N-nitroso compounds based on accurate quantitation of these experimental lesions.

Histopathological diagnoses play a crucial role in the interpretation of bioassays. Although the committee realizes that this subject falls outside the scope of its immediate charge, it believes that special efforts should be made to validate results from such diagnoses before the findings are used as a basis for decisions affecting public health.

6. Many N-nitroso compounds present in the environment or those formed from nitrate or nitrite in vivo have been shown to be carcinogenic in experimental studies. At certain dose levels, they are also acutely toxic, inducing, for example, liver damage. It is reasonable to consider that they are probably carcinogenic in humans. Therefore, the committee recommends that exposure to the precursors of N-nitroso compounds -- especially nitrate and nitrite -- and to preformed N-nitroso compounds be reduced.

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ESTIMATION OF RISK TO HUMAN HEALTH

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NOTE

Dr. Rolf Preussmann, an advisor to the Committee on Nitrite and Alternative Curing Agents in Food, does not believe that the scientific data are sufficiently reliable to be used for estimating the risk for humans exposed to N-nitroso compounds.

ESTIMATION OF RISK TO HUMAN HEALTH

Discussions in the previous chapters of this report have focused on two major issues: first, the beneficial effects of adding nitrite to foods, e.g., its ability to control the outgrowth of Clostridium botulinum spores, thereby offering protection against botulism, and second, the potential adverse effects, especially carcinogenesis, resulting from exogenous and endogenous exposure to nitrate, nitrite, and N-nitroso compounds. In this chapter, the committee discusses factors that affect the risk of botulism. In addition, it has attempted to estimate the risk of cancer that might result from exposure to nitrate, nitrite, and N-nitroso compounds and the reduction in such risk if nitrite were to be omitted from certain foods.

Quantitative risk assessment is a developing rather than a precise science. The numerical estimates in this chapter are based on a series of assumptions made by the committee. Another group using different assumptions could arrive at very different conclusions. The attempt at quantitation has been made to provide information to policymakers about the relative risks, depending on the extent of exposure to N-nitroso compounds. The absolute numbers are not meant to be a basis for policy formation.

In the discussion that follows, the committee first outlines the type of information that may be needed to estimate with accuracy the risks of botulism and cancer. Regrettably, such information is difficult to define precisely, and the available information is all too often incomplete or unreliable. This is the first hindrance to the performance of a quantitative risk assessment: the inadequacy of the data base.

The discussion proceeds to a description of mathematical models that may be used for the numerical estimation of risk. Use of these models requires a set of assumptions concerning (1) how and whether one may predict the effects of an exposure to low doses when data have been gathered from experiments performed at high doses (high to low dose extrapolation) and (2) how and whether one may predict effects in humans when data have been gathered from experiments performed in laboratory animals (interspecies conversion). Although experts may favor one model over another, they generally agree that effects may occur at lower doses and that effects observed in well-

attempted to quantitate not only the risk of carcinogenesis from exposure to nitrite and N-nitroso compounds, but also the diminution in risk if nitrite were to be removed from cured meats. The validity and limitations of the numerical estimates are discussed side-by-side in order to emphasize the uncertainty that surrounds such computations. In addition to the limitations of the various mathematical models used in estimating risks, the accuracy and reliability of the data used by the committee for human exposures from exogenously and endogenously derived nitrate, nitrite, and N-nitroso compounds are also uncertain because of the inherent weaknesses in current assay methods. Also, in some cases, averages were derived from incomplete data bases. Furthermore, accurate information on the intake of these chemicals by the U.S. population is not available.

THE RISK OF BOTULISM

Factors that Affect the Risk of Botulism

Foodborne botulism is caused by the ingestion of products in which botulinum toxin has been produced by C. botulinum. Thus, calculation of the potential risk requires consideration of two major questions: What is the likelihood that a food contains botulinum toxin? What is the likelihood that it will be ingested?

Nitrite may affect the risk of botulism by altering either or both of these probabilities. It affects the former by its inhibition of C. botulinum and may affect the latter by inhibiting other microorganisms which, had they proliferated, would have altered the palatability of the food.

The information that is needed to estimate the increase in the frequency with which foods would become toxic if nitrite were omitted from cured meats includes:

- The number, clustering, and location of spores within the contaminated product. These factors would affect the abuse time required for toxin production and the compatibility of the environment for C. botulinum growth.
- The type of spore that has contaminated the product. Different types require different minimum growth temperatures and have different growth characteristics.
- The combined effects of the treatment used and the characteristics of each type of product on control of the growth of C. botulinum and other microorganisms. Such characteristics include

- The frequency of faulty processing (e.g., insufficient level of added salt or short cooking time), which affects the ability of the specific treatments mentioned above to achieve their objective.

- The frequency with which the products are stored at suboptimal temperatures in the food distribution system, in the retail outlet, and by the consumer. For example: What proportion of which products are stored at what temperatures, and for how long? What is the proportion of retail display cabinets with temperatures higher than desirable, and by how much are they too warm? What is the proportion of home refrigerators with temperatures higher than desirable and which products are most frequently mishandled? See Bryan (1980) for a discussion of this subject.

The limited information pertaining to some of these issues (e.g. Bryan, 1980; Holley, 1978) is not sufficient to permit a calculation of the probability that a product contains toxin.

Various factors affect the likelihood that a toxic product will be eaten or that it will cause botulism if ingested.

- The type of C. botulinum contaminating the product. For example, proteolytic strains are likely to lead to a breakdown of protein and render the product aesthetically unacceptable, whereas non-proteolytic strains will not (Smith, 1977).

- The degree to which the food and drug laws are effective in facilitating the identification of potentially toxic foods and preventing them from reaching consumers. (See Johnston and Krumm, 1980)

- The method of cooking. Since botulinum toxin is sensitive to heat, the method of preparation (cooking temperature and duration) will affect the amount of toxin present at the time of ingestion (Woodburn et al., 1976).

- The dose of toxin ingested and individual susceptibility to it (Sakaguchi, 1979).

The probability of a fatality resulting from the ingestion of toxin will be dependent upon the speed of diagnosis and delivery of medical care. The number of persons that may consume a portion of toxic food is generally rather small. Between 1950 and 1977, an average of 2.4 cases were affected in an "outbreak" of botulism. However, one outbreak involved 58 cases (Center for Disease Control, 1979a,b). In recent years, there has been a decrease in fatalities attributed to botulism (Center for Disease Control, 1979a,b, 1980,

The ideal data to use in estimating the risk resulting from the omission of nitrite from foods would accrue from epidemiological studies that compare outbreaks of botulism due to consumption of meat products to which nitrite has been added and outbreaks due to consumption of similar, but nitrite-free meat products that have been handled (and abused) by producers, distributors, and consumers in an identical fashion. But no such foods exist. Thus, data of this type are not available and may never be obtainable because it is difficult to discriminate between the effects of nitrite and the effects of other contributory factors such as the pH or the salt content of the meat or the manner in which the meat has been handled.

Some Practical Experience with Reduction of Nitrate and Nitrite in Meats

Since the control of botulism is one of the objectives for the addition of nitrite to meats, it is useful to examine some recent experiences with removing nitrate and reducing the use of nitrite. Since December 1975, Norway has discontinued the addition of nitrate to cured meats and has limited residual nitrite levels to 5 mg/kg in some categories of meats to which no nitrite is added, such as fresh meats and sausages and commercially sterile canned meat items (Høyem, 1977). Fresh emulsion products to which no nitrite is added include frankfurters. These products constitute nearly one-half of the Norwegian meat market. In practice, residual nitrite is permitted in these products in concentrations up to 10 mg/kg (Nelson, personal communication, 1979). Currently, addition of nitrite is permitted in a limited number of cured products in Norway. Since 1973, when a reduction in its use was proposed, the total amount of nitrate and nitrite used as food additives in Norway has decreased by more than 80% (Ringén, personal communication, 1981). As shown in Table 5-1, a 1976 survey of Norwegian cured meat products reflects this decrease in nitrite (American Meat Institute, 1976). One large meat processor in that country has ceased to use nitrate and nitrite completely, even in products for which nitrite addition is permitted (Ringén, personal communication, 1981).

In Norway, no outbreak of botulism has been known to result from these reductions of nitrate and nitrite in cured meat products. However, caution must be exercised when applying such information to predict the impact of reducing nitrite in foods in the United States because there are several important differences between the two countries. In Norway, dietary patterns are different, the mean daily atmospheric temperature is lower, and the population is only 4 million

nitrite (see Chapter 2). Although there are some data concerning the incidence of botulism (Center for Disease Control, 1979a; Tompkin, 1980), it is difficult to compare the number of cases of botulism attributable to such products between 1899 and 1977 with the number attributable to cured products to which nitrite had been added. During much of that period, transportation and refrigeration were much less adequate than they are at present, the detection and reporting of foodborne illnesses were probably deficient, and slaughtering and processing were done on a much more local scale, thus shortening distribution chains (see Chapter 2).

Food handling and refrigeration practices in modern retail stores (Buege, 1980) and in restaurants (Bryan, 1980) are sometimes substandard. Yet, only one outbreak of botulism known to be caused by meat products either with or without nitrate- or nitrite-containing preservatives can be attributed to mishandling by these establishments (Tompkin, 1980). Panalaks *et al.* (1973, 1974) found that 108 (~36%) of 297 cured meat products analyzed contained residual nitrite in concentrations less than 7 mg/kg. Among these 108 products were numerous items in all eight product categories listed in Table 3-7. Therefore, it seems reasonable to assume that many cured meat products with low residual nitrite levels are currently on the market.

Alternative meat products that mimic cured meat, such as nitrite-free bacon, are also permitted in the United States (U.S. Department of Agriculture, 1981a,b). Although they are produced on an extremely small scale, these specialty products to which no nitrite has been added have not been known to cause an outbreak of botulism (Center for Disease Control, 1980, 1981; Tompkin, 1980).

Estimating the Risk of Botulism

Despite the lack of adequate data, efforts have been made to determine the increment in the risk of botulism (e.g., the extra number of cases or deaths that might result) if nitrite and nitrate were no longer used in cured products. The Nitrite Task Force of the Food and Drug Administration (1979a) attempted to estimate the increased risk of botulism that is likely to result from the omission of nitrite from cured meats. This group used data on the observed number of deaths from the outbreaks of botulism caused by the consumption of commercially processed smoked fish containing type E *C. botulinum* in the early 1960's. By applying various factors to adjust for the relative amount of cured meats (as compared to smoked fish) consumed, differences in the prevalence and types of *C. botulinum* spores in the products, and food handling practices of smoked fish

certainly about the validity of the assumptions, and the questionable basis for the calculations.

In order to use epidemiological data to assess the absolute risk of botulism or reduction in risk due to the addition of nitrite and other ingredients in the curing process, it is essential to obtain reliable data on the incidence of or mortality from botulism. Despite the existence of a sophisticated system for reporting botulism in the United States, the difficulty in diagnosing this disease (Center for Disease Control, 1979a; Sakaguchi, 1979) would lead one to suspect that cases of botulism are misdiagnosed and attributed to other causes. Microbiological characterization of C. botulinum or botulinum toxin during autopsy of cases of sudden and unexpected death could assist in determining if misdiagnoses contribute significantly to the underreporting of botulism. Such concerns were raised by the results of an unconfirmed study conducted in Switzerland (O. Sonnabend, 1981; W. Sonnabend, 1981). The Center for Disease Control (1979a,b, 1980, 1981) has reported that only three deaths due to botulism have been traced to ingestion of fresh meats or commercially produced meat and poultry products in the United States during the past 55 years.

Discussion and Conclusions

The critical effect of nitrite in combating botulism is its inhibition of the outgrowth of C. botulinum spores. However, other factors, e.g., salt, pH, and thermal processing, also contribute to the suppression of spore outgrowth, cell growth, and, thus, toxin production. If a product is not stored at optimal temperatures, the contribution of nitrite to the suppression of spore outgrowth varies with the type of product and other conditions prevalent at the time of the abuse (see Chapter 3). However, there are no data on its impact on the entire range of variables in any one product, on these variables in all classes of products, or in different situations in which the product may be abused.

Because of the lack of adequate information on the frequency of various events in the sequence leading to the production of botulinum toxin and, thus, botulism, e.g., contamination and length and conditions of abuse, it is impossible to predict the likelihood of products becoming toxic or becoming sufficiently unpalatable to be ingested. Thus, although nitrite prolongs the period that a contaminated product can be stored at suboptimal temperatures without becoming toxic, it is impossible to predict the extent to which this extended endurance reduces the incidence of botulism. Moreover, epidemiological data cannot be used to determine the increased risk of botulism that might result if nitrite were omitted from products to which it is currently

THE BIOLOGICAL BASIS FOR ESTIMATING THE RISK OF CANCER FROM THE ADDITION OF NITRATE AND NITRITE TO FOODS

As discussed in earlier chapters of this report, humans are exposed to nitrate, nitrite, nitrogen oxides, and N-nitroso compounds in the diet and in other environmental media. Since nitrate, nitrite, and nitrogen oxides can react with other substances in the diet and in the human body to produce N-nitroso compounds, most of which produce cancer in animals, it is desirable to determine the increased risk arising from their addition to foods as well as the risk posed by their natural occurrence in foods. These risks could be estimated with confidence if there were accurate information on exposure to these substances, if their metabolism and pharmacokinetics in humans were well understood, and if the total body burden of N-nitroso compounds were known.

In order to estimate exposure to nitrate, nitrite, and N-nitroso compounds, it is necessary to know:

- The average and the range of the amounts of nitrate and nitrite ingested, the nature and size of the population subgroups consuming different amounts, and the proportion of the intake resulting from deliberate addition of these compounds to foods.
- The characteristics and sizes of groups with high intakes of nitrosatable substrates such as certain drugs.
- Individual variability in the metabolism of these compounds, e.g., in salivary nitrate reduction or endogenous synthesis of nitrate.
- The amount and pattern of nitrate, nitrite, and nitrosatable substrates in the stomach at any one time in healthy and diseased individuals; the physiological conditions in the stomach; and the probability for the generation of N-nitroso compounds (as discussed by Ohshima and Bartsch, 1981). Or, alternatively, experimental evidence pertaining to the amounts and types of all N-nitroso compounds produced in the normal and abnormal stomach over a wide range of intakes and dietary patterns.
- The sizes of population subgroups with abnormal stomach conditions or other gastrointestinal disorders.
- Exogenous and endogenous exposure to other nitrosating agents, e.g., nitrogen oxides and preformed nitrosamines, and to the modifiers of nitrosation, e.g., thiocyanate, ascorbic acid, and α -tocopherol.

pair mechanisms for each of the N-nitroso compounds.

- The tissue specificity, latent period between exposure and tumor formation in humans exposed at various ages, and the carcinogenic potency of each N-nitroso compound formed in vivo.

- The fatality rate of the various types of tumors induced (if the risk is to be expressed as possible deaths).

Discussion and Conclusions

The major sources of environmental exposure to nitrate, nitrite, and N-nitroso compounds have been specified and, to the extent possible, quantified in Chapters 5, 6, and 7 (see Tables 5-20, 5-21, 6-1, and 7-17). As pointed out earlier, the data base is clearly limited and considerable uncertainty surrounds any attempt to assign confidence limits to these estimates. Similarly, Chapter 8 summarizes the current knowledge pertaining to metabolism and pharmacokinetics and provides estimates of the endogenous exposure to nitrate, nitrite, and N-nitroso compounds (Tables 8-3 and 8-4). It is apparent that nitrate and nitrite can participate in the formation of N-nitroso compounds. It also appears that nitrosation (kinetically correlated with in vitro experiments) can occur in the human body (Ohshima and Bartsch, 1981) and that the process can be blocked by ascorbic acid and α -tocopherol (see Table 8-3). However, little is known about the nitrosating potential of naturally occurring nitrate and nitrite and of nitrite added to cured meats, and knowledge concerning the formation of various N-nitroso compounds in humans is extremely limited. Moreover, differences in the rates of metabolism and the potency of these compounds as tested in animals and the variability in individual susceptibility to their effects make it difficult to provide reliable estimates of their total body burden and, consequently, the risk to human health.

Nonetheless, bearing these limitations in mind and using a number of clearly delineated assumptions, the committee has attempted to compute the risk of cancer for humans by using data on tumor incidence from two experiments in which rats were fed a nitrosamine, and discusses the feasibility of using data from one in which mice were given nitrite and an amine. In another series of calculations to assess the risk of carcinogenesis and mortality for various population groups, the committee used data on tumor incidence in animals; estimates of exogenous exposure to nitrate, nitrite, and N-nitroso compounds; and estimates of total exposure to N-nitroso compounds

were removed from cured meats by assuming certain well-delineated characteristics of exposure for these population groups.

The assumptions and methodology used in these computations and their limitations are the focus of the next section.

ESTIMATION OF THE RISK OF CANCER FOR HUMANS EXPOSED TO NITRATE, NITRITE, AND N-NITROSO COMPOUNDS

This section has been prepared primarily for use by those readers who are familiar with quantitative risk assessment and who may wish to compare the methodology used by the committee with other methods.

The risks estimated in this chapter should be regarded as rough approximations of doses that might be relatively innocuous for humans. It would be misleading to equate these estimates with predictions derived from data obtained from the dose ranges actually used in the animal experiments.

Quantitative Risk Assessment Based on Lifetime Feeding Studies on Animals

In this report, quantitative risk assessment refers specifically to the estimation of the probability or the risk that various toxic end points will result from a range of doses or levels of exposure over a lifetime. The rationale and the details of the methodology for such assessments can be found in a recent report prepared by the Scientific Committee of the Food Safety Council (Food Safety Council, 1980) and in many papers (e.g., Cornfield, 1977; Cornfield et al., 1978; Crump et al., 1977; Hartley and Sielken, 1977; Hoel et al., 1975; Krewski and Van Ryzin, in press; Mantel and Bryan, 1961; Rai and Van Ryzin, 1979, 1981; Van Ryzin, 1980).

Data from lifetime feeding studies are generally derived from a group of animals (n_0) tested at dose $d_0 = 0$, i.e., a control group, and groups of animals (m) tested at doses $d_1 < d_2 < \dots < d_m$ with n_1, n_2, \dots, n_m animals, respectively. The toxic responses, $x_0, x_1, x_2, \dots, x_m$, are recorded for each dose for each toxic end point. For example, x_i = the number of animals exhibiting the toxic end point under study at dose d_i , where $i = 0, 1, 2, \dots, m$. Using these data, one can attempt to predict the probability that such a toxic end point will occur in humans at doses to which they might be exposed.

the resulting estimate of a dose corresponding to a specified risk.

A dose-response model is a mathematical equation that relates the dose, d , to the rate of occurrence (incidence) of response or the probability (P) that an animal exposed to that dose will exhibit a specific toxic end point during its lifetime. This function is typically written as $P(d)$ (Figure 10-1).

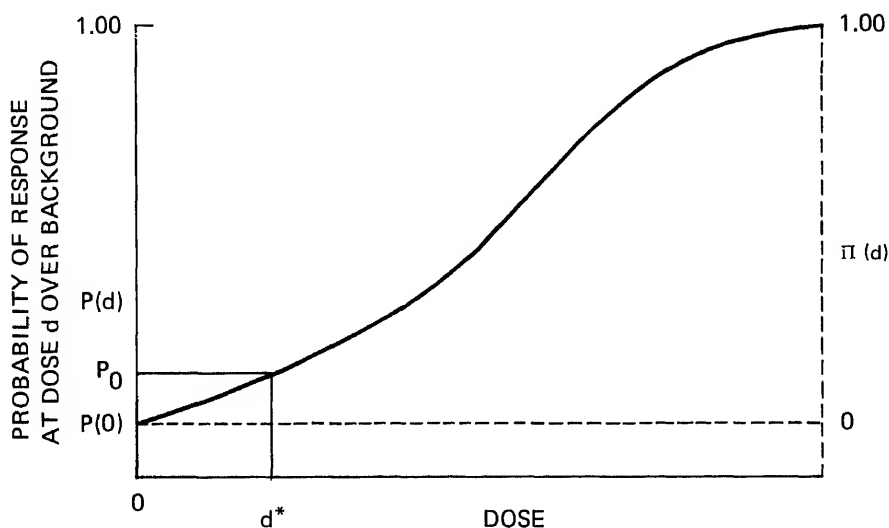


FIGURE 10-1. A dose-response curve. P_0 = specific level of risk; $P(0)$ = background or spontaneous response rate; d^* = the dose that corresponds to a specified risk; $\Pi(d)$ = probability of toxic response at dose d .

The quantity $P(0)$ represents the background or the spontaneous rate of response. $P(d)$ is usually assumed to be an increasing function of d , reflecting the concept that an increase in the dose of a toxic substance increases the probability of a toxic response.

The specified level of risk is based on the probability of a toxic response at dose d , minus the spontaneous rate of response $P(0)$, divided by the probability of no response from the background:

the background exposure. In Figure 10-1, the curve $\Pi(d)$ is represented by the vertical dashed line. For toxic end points such as liver tumors, the specified risk level, P_0 , may be 10^{-6} , which corresponds to a frequency of approximately three cases per year in the United States. If one assumes that approximately 220 million people whose average lifespan is approximately 72 years are exposed annually at this level, this would lead to an effective rate of 3 million people per year at risk, i.e., $3 = (10^{-6} \times 3,000,000)$ cases of cancer per year. Once the specified risk level, P_0 , is known, the equation $\Pi(d^*) = P_0$ yields the corresponding dose level d^* ¹. The dose d^* is that level which would, for the average subject exposed to a chronic dose d^* (usually expressed as mg/kg body weight/day, %, or ppm), result in an increased risk of P_0 over the background level of risk. This dose d^* is illustrated in Figure 10-1.

The statistical problem of extrapolating from high to low dose can now be easily described. By using a suitable statistical estimation technique, usually the maximum likelihood estimation, estimates of the function $P(d)$, denoted $\hat{P}(d)$, and the background $P(0)$, denoted by $\hat{P}(0)$, can be obtained. These lead to an estimate of $\Pi(d)$, given by the equation $\hat{\Pi}(d) = [\hat{P}(d) - \hat{P}(0)] / [1 - \hat{P}(0)]$. This estimate of $\hat{\Pi}(d)$ depends on all the response data given by the observed rates of response $x_0/n_0, x_1/n_1, \dots, x_m/n_m$ at doses d_0, d_1, \dots, d_m , respectively. Having estimated this, one can estimate the resulting dose, denoted by \hat{d}^* , by solving the equation $\hat{\Pi}(\hat{d}^*) = P_0$. The estimated dose \hat{d}^* for a specific risk P_0 based on the actual data from the experiment represents the experimentally determined estimate of that dose which would lead to an increased risk of P_0 . This process is shown in Figure 10-2, where $\Pi(d)$, the dashed line, represents the true unknown dose-response curve, and $\hat{\Pi}(d)$, the solid line, represents the estimate of that curve based on the data. The figure has been included for illustrative purposes only; $\hat{\Pi}(d)$ is not always less than $\Pi(d)$ in the low dose region.

¹The dose d^* , which corresponds to a specific risk $P_0 = 10^{-8}$ when combined in the probit model with an arbitrary slope of unity, was called the "virtually safe dose" by Mantel and Bryan (1961). The committee chose not to use the word "safe" because it may be understood differently by different people. For example, if the chance of a skiing accident is 1 in 10,000, that may be safe to one person, unsafe to another. "Safety" is a regulatory/political/societal decision, which scientists alone cannot determine.

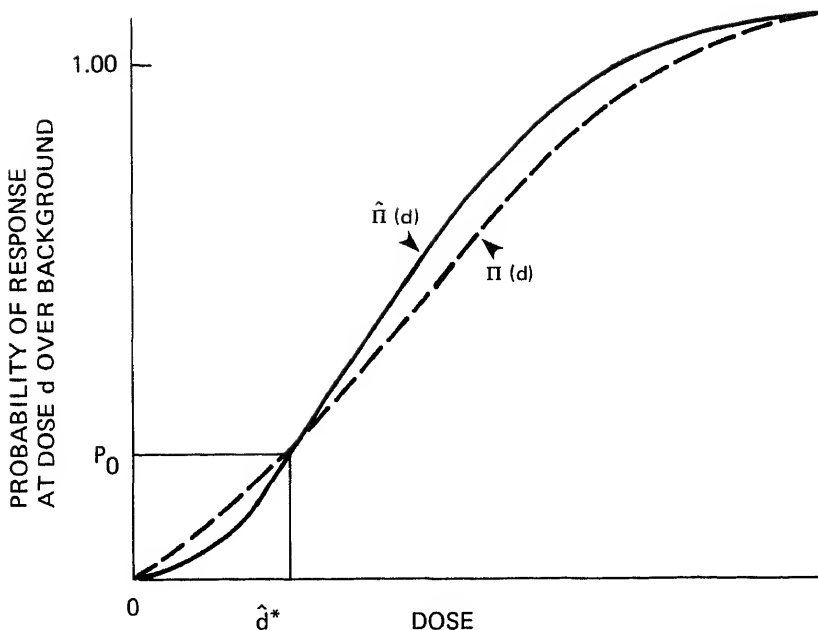


FIGURE 10-2. Calculation of a dose \hat{d}^* that corresponds to a specified risk, P_0 . $\Pi(d)$ = probability of toxic response at dose d ; $\hat{\Pi}(d)$ = estimate of dose-response curve.

Note that this process of estimating the dose d^* for a specific risk involves certain statistical uncertainties since it depends on data that vary from one experiment to another. There is no one mathematical model that is best for the estimation of risk to humans. Therefore, a further, and perhaps far more serious source of error, is that these estimates of dose will vary considerably, depending on the mathematical model used for the dose-response function $\Pi(d)$ and the manner in which the background response is incorporated. Because of the uncertainty of the risk estimates based on mathematical models, the committee has based its calculations of these doses on three mathematical models: the one-hit model, the multistage model, and the multihit model.

Each of the models is described in terms of the function $\Pi(d)$.

The method used in equation (1) for incorporating the background response, $P(0)$, was first described by Abbott (1925), and is known as Abbott's correction. That equation assumes that the response due to the background exposure is statistically independent of the response due to the additional dose. That is, equation (1) may be rewritten as:

$$1 - P(d) = [1 - P(0)] [1 - \Pi(d)], \quad (2)$$

which states that the probability that there is no toxic response at dose d is the product of the probability of no response from background exposure and the probability of no response from the additional dose. This is an important assumption because it means that the outcome at the low doses represented by the dose-response curve will be determined by the mathematical model adopted for $\Pi(d)$. However, suppose that equation (1) is not assumed, but, instead, one assumes that the additional dose reacts additively with a postulated "effective" background dose, $d_0 > 0$ (Crump et al., 1976; Peto, 1978). Specifically suppose that:

$$P(d) = f(d + d_0) \quad (3)$$

for a given dose-response function, $f(d)$. Then the shape of the curve for the incremental risk function, $\Pi(d) = [P(d) - P(0)] / [1 - P(0)]$, will be approximately linear for small additional doses if the slope of the curve for function f at d_0 is positive. That is, for small doses, it is mathematically easy to show that:

$$\Pi(d) = \text{approx. } c \text{ or } d, \quad (4)$$

where $c = f'(d_0) / [1 - P(0)]$, and $f'(d_0) > 0$ is the first derivative or slope of f at d_0 . The function $f'(d_0)$ would always be positive if the underlying dose-response curve $f(d)$ continues to increase and has no threshold, i.e., there is no point $d' > 0$, or there is no value greater than zero on the dose scale. Thus, $f(d) = 0$ if $d \leq d'$.

Equation (4), known as low dose linearity, has important consequences for high to low dose extrapolation. Its implications for

$$\Pi_1(d) = 1 - e^{-\theta d}, \quad (5)$$

where $\theta > 0$ is a constant.

Multistage Model:

$$\Pi_2(d) = 1 - e^{(\theta_1 d + \theta_2 d^2 + \dots + \theta_k d^k)}, \quad (6)$$

where $\theta_1 \geq 0$, $\theta_2 \geq 0$, ..., $\theta_k \geq 0$ are constants and k = number of stages.

Multihit Model:

$$\Pi_3(d) = \int_0^{\theta d} (u^h - 1) e^{-u} du / \Gamma(h) \quad (7)$$

where $\theta > 0$ is a constant, h is the number of "hits", and the gamma function is $\Gamma(d) = \int_0^\infty (u^h - 1) e^{-u} du$, where u is the variable of integration.

The mathematical reasoning for each of these models is not discussed here. For detailed descriptions of the one-hit model, see Hoel et al. (1975) or Rai and Van Ryzin (1979); for the multistage model, see Armitage and Doll (1961) and Crump et al. (1976); and for the multihit model, see Rai and Van Ryzin (1979, 1981). Each model is based on various biological assumptions, which are explained in the papers cited. A critical variable in high to low dose extrapolation is the applicability of each of these equations at low doses. Depending on the value of the constants, this variability in applicability at low doses can be severe. For low doses, whenever $k > 1$ and $h > 1$, then:

$$\Pi_1(d) > \Pi_2(d) > \Pi_3(d), \quad (8)$$

when $k = h = 1$, $\Pi_1(d) = \Pi_2(d) = \Pi_3(d)$ = equation (5) for all doses, and all three models are identical. Equation (8) is important because if \hat{d}_1^* , \hat{d}_2^* , and \hat{d}_3^* represent estimates of the dose corresponding to a small specified level of risk P_0 , it can be shown in most cases that:

analyzed in approximately 20 data sets by Krewski and van Kyzyn (in press). These authors discussed the three models mentioned here along with three additional dose-response models: the logistic, Weibull, and probit models. They demonstrated that the results of the Weibull and logistic models are usually close to \hat{d}_3^* , whereas that of the probit model exceeds \hat{d}_3^* . Thus, for the dose estimates presented in this report, the relative order illustrated in equation (9) will suffice for our purposes. At very low specified levels of risk, the spread between \hat{d}_1^* and \hat{d}_3^* may often be several orders of magnitude.

In addition to using different data sets to estimate \hat{d}^* at various levels of risk for the three models, the committee has calculated the "goodness-of-fit" of the experimental data for each of the models. Also provided are the results of a statistical test to determine if the one-hit model fits the data. It is important to evaluate all this information when incorporating the assumed degree of linearity into quantitative risk assessments.

As mentioned above, the one-hit model always leads to a linear extrapolation for low doses, typically providing higher estimates of risk and lower estimates of doses than the other models. Whether such estimates are accurate depends, in part, on how the background rate of response interacts with the increased rate of response. As mentioned earlier, if this inaction is additive for the range of doses used, the assumption that the response will be linear at low doses is appropriate [equations (3) and (4)].

Estimates of risk assuming low dose linearity may be a prudent course, regardless of the statistical considerations. However, the role of DNA repair mechanisms at low doses should also be considered when making such estimates. For example, when the biological evidence indicates that a carcinogen under study may act through direct-acting mechanisms such as DNA binding, rather than as a promoter or modifier, it may be prudent to use linear extrapolation, regardless of the shape of the dose-response curve (Chapter 11 in Food Safety Council, 1980). When there is little or no support for dose-wise additivity for direct-acting genotoxic substances, estimates based on the multihit model or the multistage models may be more appropriate because both models allow for nonlinearity. These models will generally result in a steep dose-response curve, leading to estimates of dose at specified levels of risk that are higher than those obtained with the one-hit model.

Doses for each of the data sets are estimated for risk levels of $P_0 = 10^{-2}, 10^{-4}, 10^{-6}$, and 10^{-8} for each of the three models. In addition, the estimates of the dose to correspond to a risk of 10^{-2} for the multistage and multihit models are multiplied by factors of $10^{-2}, 10^{-4}, 10^{-6}$. Such estimates of the dose are linearized model esti-

gives an upper bound on the risk and, hence, a lower bound on the dose. This is illustrated in Figure 10-3 for a risk of $P_0 = 10^{-6}$.

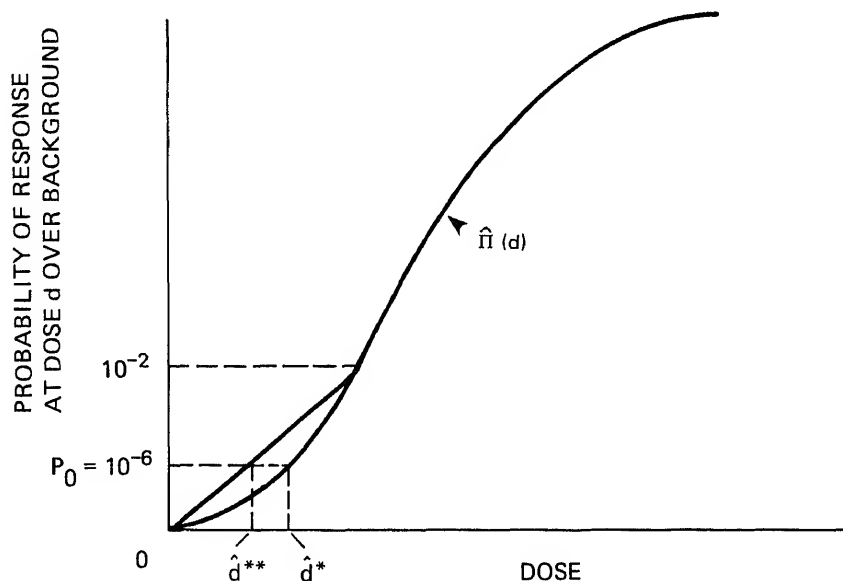


FIGURE 10-3. Linearized model estimation for $\hat{\Pi}(d)$ (probability of toxic response at dose d) at 10^{-6} . \hat{d}^* = dose at 10^{-2} extrapolated from $\hat{\Pi}(d)$, \hat{d}^{**} = dose at 10^{-6} extrapolated from $\hat{\Pi}(d)$ to 10^{-2} , followed by linear extrapolation to 10^{-6} . $\hat{\Pi}(d)$ = estimate of convex dose-response curve.

An important point to be recognized is that estimates of doses at various levels of risk are based on data from tests in animals and represent doses that correspond to various levels of risk for the species of animal studied. The relevance of these data for humans is addressed below.

Recently, attention has been focused on risk extrapolation models that relate the probability of tumor induction to both the dose and the time-to-tumor occurrence (Hartley and Sielken, 1977). These models are important because time-to-tumor occurrence increases with reduction in dose (Druckrey, 1967). Thus, the lower doses may be less capable of inducing tumors during the animal's lifetime than

tumors (Society of Toxicology Task Force Report, 1981). Unfortunately such models and risk assessment cannot be applied to N-nitroso compounds because there are no adequate data.

Interspecies Conversion. The estimates of the dose for specified levels of risk that are given later in this chapter are presented in dose units used in the original animal experiment, either in mg/kg body weight per day or ppm (mg/kg) in the diet on a dry weight basis, and they apply to the species of animal used in the experiment. To relate these estimates of dose to exposures of humans, it is necessary to do an interspecies conversion. Two methods commonly used for these conversions are:

- Conversion to mg/kg body weight per day. Concentrations of test substances in the animal's diet are usually expressed as ppm or ppb, which is equal to mg/kg and $\mu\text{g/kg}$ of diet, respectively. The concentration in the original experiment is multiplied by the weight of the diet consumed by the animal daily and then divided by the weight of the animal. This amount (milligrams of test substance per kilogram of body weight per day) is then used directly as a measure of the exposure of humans expressed as mg/kg body weight per day.

- Surface area conversion. The mg/kg body weight dosage given to the animal species is converted to mg/cm^2 body surface area. Exposure of humans is then given in equivalent terms. This type of conversion is often used in cancer chemotherapy and was the method of conversion adopted by the Safe Drinking Water Committee (National Academy of Sciences, 1977).

Opinions differ as to which of the two methods of conversion is preferable. Therefore, both sets of calculations are presented in this report.

Having discussed in general terms the methodology for estimating a dose corresponding to a specific risk, P_0 , the committee has devoted the next two sections to applying this methodology to data from experiments in animals for two N-nitroso compounds (nitrosodimethylamine and nitrosopyrrolidine) and for nitrite in the presence of a fixed level of an amine (piperazine). Following these discussions, the results are summarized and their meaning for risks to humans exposed to nitrate and nitrite is examined. The risk estimates for N-nitroso compounds are then combined with data on the exposure of humans to nitrate and nitrite to arrive at estimates of risk for specific subgroups of the population.

two sets of data containing sufficient dose-response information to fit all three models with $k > 1$ for the multistage model and $h > 1$ for the multihit model. At a minimum, this means we are restricted to data sets with $m > 2$. If $m = 1$, then $k = h = 1$, and only the one-hit model can be used to estimate risk. Thus, extrapolations can be avoided.

Also, because of uncertainty in the estimation procedure whenever the data are insufficient to indicate a statistically significant increase in the dose-response function, it makes little sense to force fit statistics for dose-response functions that continue to increase with dose, such as those given by the functions $\Pi_1(d)$, $\Pi_2(d)$, and $\Pi_3(d)$. Specifically, for all the data sets given in this section and the next one on nitrite, a test for statistical significance for trend was conducted before risk assessment models were used. That is, a test was conducted to determine the justification of $P(0) < P(d_1) < \dots < P(d_m)$ -- an increasing dose-response model. The statistical test, described by Mantel (1963) and Tarone (1975), was carried out to a 5% level of significance in all cases.

Nitrosodimethylamine (NDMA)

The dose-response data in Table 10-1 pertain to liver tumors in rats fed daily doses of NDMA in a chronic toxicity test (Terracini *et al.*, 1967). For this data set, the estimated doses corresponding to risk levels $P_0 = 10^{-2}$, 10^{-4} , 10^{-6} , and 10^{-8} are given for the one-hit, multistage, and multihit models in Table 10-2. The figures in parentheses in Table 10-2 are the linearized model estimates at $P_0 = 10^{-4}$, 10^{-6} , and 10^{-8} for the multistage and multihit models.

In addition to the results in Table 10-2, the estimated constant, h , for the multihit model was $\hat{h} = 1.57$, where ± 0.35 is the standard error of the estimate. The multistage model resulted in a two-stage model for which θ_1 and θ_2 were estimated to be: $\theta_1 = 0.0236$ and $\theta_2 = 0.000521$. All three models had acceptable "goodness-of-fit" statistics at a level of significance of 0.05. Thus, each of the models provides a reasonably good fit in the experimental range for the positive dose levels. The orders of magnitude differences in the estimates at 10^{-4} through 10^{-8} can be explained by the different sensitivity of the models in the low dose range. As suggested in equation (9), the ordering of dose estimates is $d_1^* < d_2^* < d_3^*$.

Finally, a statistical likelihood ratio test of the hypothesis that $h = 1$ (a one-hit model and low dose linearity) versus $h > 1$ (nonlinearity) (Rai and Van Ryzin, 1981) yields a chi-squared value of 3.82 which for one degree of freedom has an observed significance

<u>Group (i)^b</u>	<u>Dose (d_i) of NDMA in the Diet, ppm</u>	<u>No. of Animals with Liver Tumor (x_i)</u>	<u>No. of Animals on Test (n_i)</u>
0	0	0	41
1	2	1	37
2	5	8	83
3	10	2	5
4	20	15	23
5	50	10	12

^aData from Terracini et al., 1967.

^bThe first three groups contained males and females; the last three contained females only. The 5 ppm dose was given to two groups of animals, which are combined as Group 2 in this table. Exclusion of males from the calculations would lead to lower estimates of risk and a higher estimate of doses at the various risk levels in Tables 10-2 and 10-3.

TABLE 10-2

Estimated Daily Doses of N-Nitrosodimethylamine (NDMA) Corresponding to Specific Levels of Risk of Liver Tumors in Rats^a

<u>Model</u>	<u>NDMA in Diet, ppb, by Level of Risk (P₀)</u>			
	<u>10⁻² (1/100)</u>	<u>10⁻⁴ (1/10,000)</u>	<u>10⁻⁶ (1/million)</u>	<u>10⁻⁸ (1/100 million)</u>
One-hit (\hat{d}_1^*)	303	3.0	0.03	0.0003
Multistage (\hat{d}_2^*)	421	4.2 (4.2) ^b	0.04 (0.04)	0.0004 (0.0004)
Multihit (\hat{d}_3^*)	974	51.0 (9.7)	2.7 (0.097)	0.15 (0.00097)

committee assumed linearity below $P_0 = 10^{-2}$, extrapolated for levels of risk below 10^{-2} with the upper bound on risk in accordance with Figure 10-3, and used these linearized estimates at 10^{-4} , 10^{-6} , and 10^{-8} . This approach is cautious in that it assumes low dose linearity below a risk level of 10^{-2} (0.01) and extrapolates only slightly beyond the observed frequency level 0.027 (1/37) at the lowest observed positive dose level of 2 ppm (2 mg/kg). The multihit and multistage models were used.

The committee used the numbers in parentheses for the multihit model in Table 10-2 as estimated doses corresponding to specific levels of risk for the species of animal tested (in this experiment, the rat). It then converted these estimates to the daily dose levels for humans and expressed them as mg/kg body weight and mg/cm² body surface. Paget (1965) reported that the ratio of the dose in mg/kg body weight to dose in mg/cm² surface area for rats is 1.43, whereas for humans, it is 9.8. Therefore, the data from rats were converted to body surface area for humans by dividing the data in the first row of Table 10-3 by 6.85 (i.e., 9.8/1.43) to obtain the estimates in the second row.

N-Nitrosopyrrolidine (NPYR)

The dose-response data in Table 10-4 pertain to malignant liver tumors in rats fed NPYR (Preussmann et al., 1977). In applying the models to these data, there was a lack of fit at the level of significance of 0.05 for each of the models when the highest dose was included. This is primarily because there was a decreased response rate of 37.5% (9/24) at that dose (10.0 mg/kg body weight) as compared to 81.5% (31/38) at the second highest dose (3.0 mg/kg body weight). The usual practice when such reversals occur at the highest dose is to exclude that dose when analyzing the data. With this adjustment, the tests of Mantel (1963) and Tarone (1975) show a definite increase in dose response at the 5% level of significance for the data in Table 10-4.

For the data set in Table 10-4 (excluding the highest dose), the doses that correspond to risk levels $P_0 = 10^{-2}$, 10^{-4} , 10^{-6} , and 10^{-8} are given in Table 10-5 for the one-hit, multistage, and multihit models. The figures in parentheses in that table are the linearized model estimates at $P_0 = 10^{-4}$, 10^{-6} , and 10^{-8} for the multistage and multihit models. The estimated constant, h , for the multihit model was $\hat{h} = (3.14 \pm 0.74)$, and the multistage model resulted in a two-stage model with θ_1 and θ_2 estimated to be $\hat{\theta}_1 = 0$ and $\hat{\theta}_2 = 0.19635$. Both the multistage and multihit models had acceptable "goodness-of-fit" statistics at a level of significance of 0.05, whereas there was a

TABLE 10-3

Estimates for Daily Doses of N-Nitrosodimethylamine (NDMA) that Correspond to Specific Levels of Risk for Humans^a, Based on Multihit Model with Linear Extrapolation from $P_0 = 10^{-2}$

<u>Basis for Estimate</u>	<u>Doses of NDMA, by Level of Risk (P_0)</u>			
	<u>10^{-2}</u>	<u>10^{-4}</u>	<u>10^{-6}</u>	<u>10^{-8}</u>
ng/kg body weight/day	58,400	584	5.84	0.058
mg/cm ² body surface/ day ^c	8,525	85.3	0.85	0.0085

^aBased on data from Terracini *et al.*, 1967.

^bUsing 97.4 ng/kg (0.0974 ppb) of NDMA in the diet, and assuming the average daily diet for rats to be 0.015 kg and the average weight of a rat to be 0.25 kg, the amount in ng/kg body weight daily would be 5.84. That is: $\frac{97.4 \times 0.015}{0.25} = 5.84$ ng/kg.

^cThe ratio of the weight of the diet (mg) to body surface area (cm²) for rats is 1.43 and for humans 9.8 (Paget, 1965). Thus, the ratio 6.85 (9.8/1.43) is the factor used to convert the numbers in row 1 to row 2. That is, the numbers in row 2 are obtained by dividing the numbers in row 1 by 6.85.

<u>Group (i)</u>	<u>Dose (d_i) of NPYR in Diet (mg/kg of Body Weight/Day)</u>	<u>No. of Animals with Malignant Liver Tumors (x_i)</u>	<u>No. of Animals on Test (n_i)</u>
0	0.0	0	61
1	0.3	0	60
2	1.0	13	62
3	3.0	31	38
4	10.0	9	24

^aData from Preussmann et al., 1977.

Therefore, the multistage and multihit models give a reasonably good fit in the experimental range for the positive dose levels. The differences in orders of magnitude in the extrapolations, especially at 10^{-4} , 10^{-6} , and 10^{-8} , are due to the different sensitivity of the models in the low dose range. As suggested in equation (9), the ordering of dose estimates is $\hat{d}_1^* < \hat{d}_2^* < \hat{d}_3^*$.

A statistical likelihood ratio test of the hypothesis $h = 1$ (a one-hit model and linearity) versus $h > 1$ (multihit and nonlinearity) (Rai and Van Ryzin, 1981) yields a chi-squared value of 18.46 for one degree of freedom, which is significant at the 0.001 level. Thus, there is strong evidence for nonlinearity in the observed range. A similar result is obtained in a test that rejects $\theta_1 = 0$ (and hence linearity) in the multistage model using the results of Crump et al. (1977). Thus, the question of what estimates seem reasonable for low dose extrapolation and risk assessment is more difficult to answer in this case where linearity in the observed dose-response curve is not substantiated by the data.

The committee used two methods of extrapolation, both of which provide a conservative estimate of the dose that corresponds to a specific risk. In the first method, the linearized estimates for

to specified levels of risk for liver tumors in rats. Estimates are Based on Data in Table 10-4, with the Highest Dose Excluded^a

Model	<u>Doses of NPYR, mg/kg Body Weight/Day, by Level of Risk (P₀)</u>			
	<u>10⁻²</u>	<u>10⁻⁴</u>	<u>10⁻⁶</u>	<u>10⁻⁸</u>
One-hit (\hat{d}_1^*)	3.0×10^{-2}	3.0×10^{-4}	3.0×10^{-6}	3.0×10^{-8}
Multistage (\hat{d}_2^*)	2.3×10^{-1}	2.3×10^{-2} (2.3×10^{-3}) ^b	2.3×10^{-3} (2.3×10^{-5})	2.3×10^{-4} (2.3×10^{-7})
Multihit (\hat{d}_3^*)	3.1×10^{-1}	6.5×10^{-2} (3.1×10^{-3})	1.5×10^{-2} (3.1×10^{-5})	3.4×10^{-3} (3.1×10^{-7})

^aBased on data from Preussmann *et al.*, 1977.

^bNumbers in parenthesis are the linearized estimates.

the multihit model, where $1.92 = 3.14 - (1.65)(0.74)$, and uses this value of h for extrapolation with the same model. The first method (A) forces low-dose linearity, whereas the second method (B) allows for a minimal amount of low dose nonlinearity, as suggested by the 95% lower confidence limit on h. Results calculated by both methods are presented in Table 10-6 as unit of dose per unit of body weight and per unit of surface area for risk levels 10^{-2} , 10^{-4} , 10^{-6} , and 10^{-8} .

Comparison of Tables 10-5 and 10-6 indicates that there are considerable differences among dose estimates, especially at the 10^{-6} and 10^{-8} levels, depending on the choice of model and extrapolation method adopted (e.g., linearized, lower confidence limits on certain key parameters, etc.).

Risk Extrapolations for Nitrite Plus Amine. There are no reliable dose-response studies in which tumor induction has been observed to increase with the dose of nitrite alone. The Interagency Working Group on Nitrite Research (Food and Drug Administration, 1980) reanalyzed an earlier study in which rats were fed different doses of sodium nitrite over a 2-year period (see Chapter 9). No
ig ca do e-r sponse cr ve r u ted f om hi study. Accordingly,

Basis of Estimate and Model Used	Doses of NPYR, by Level of Risk (P_0)			
	10^{-2}	10^{-4}	10^{-6}	10^{-8}
<u>ng/kg Body Weight/Day:</u>				
Method A ^b	3.1×10^5	3,100	31	0
Method B ^c	8.3×10^4	7,220	652	59
<u>mg/cm² Body Surface/Day:^d</u>				
Method A	4.5×10^4	452	4.5	0
Method B	1.2×10^4	1,054	95	8

^aBased on data from Preussmann et al., 1977.

^bMethod A: Multihit with linear extrapolation from $P_0 = 10^{-2}$.

^cMethod B: Multihit with 95% lower confidence limit on h.

^dSee footnote c of Table 10-3 for basis of calculation.

the committee has considered an experiment by Greenblatt and Mirvish (1973), in which Strain A mice received a diet containing 6 g piperazine per kilogram of diet and drinking water containing various concentrations of sodium nitrite. The mice received the treatment for 20 weeks, beginning at 10 weeks of age, and were killed when they were 40 weeks old. The surface lung adenomas were then counted. The results were expressed as percent incidence of tumors and as number of tumors per mouse, i.e., tumor multiplicity. The authors found that the yield of tumors per mouse was proportional to the square of the concentration of nitrite. However, since tumor multiplicity is not considered in the mathematical models used to obtain risk estimates, the committee has used the data on tumor incidence (Table 10-7).

Direct risk extrapolation for nitrite and a nitrosatable amine (piperazine) from studies in animals fed for 1 to 40 weeks is shown in Table 10-8 for the one-hit, multistage, and multihit models at risk levels 10^{-2} , 10^{-4} , 10^{-6} , and 10^{-8} . The figures in parentheses are the linearized model estimates for risk levels 10^{-4} , 10^{-6} , and 10^{-8} for the multihit model.

<u>Group (i)</u>	<u>Dose of Sodium Nitrite in water (g/liter) (d_i)</u>	<u>No. of Mice with Lung Adenoma (x_i)</u>	<u>No. of Mice on Test (n_i)</u>
0	0	11	39
1	.05	10	39
2	.25	20	40
3	.50	30	39
4	1.0	37	37
5	2.0	39	40

^aData from Greenblatt and Mirvish, 1973.

TABLE 10-8

Estimated Daily Doses of Sodium Nitrite in Drinking Water Which, in the Presence of 6 g of Piperazine per Kilogram of Diet, Correspond to Various Levels of Risk of Lung Adenomas in Strain A Mice^a

<u>Model</u>	<u>Dose of Sodium Nitrite, mg/liter Drinking Water, Level of Risk (P_0)</u>			
	<u>10^{-2}</u>	<u>10^{-4}</u>	<u>10^{-6}</u>	<u>10^{-8}</u>
One-hit (\hat{d}_1^*) and Multistage (\hat{d}_2^*); $\hat{d}_1^* = \hat{d}_2^*$	4.3	4.2×10^{-2}	4.2×10^{-4}	4.2×10^{-6}
Multihit (\hat{d}_3^*)	17	8.5×10^{-1} (1.7×10^{-1}) ^b	4.2×10^{-2} (1.7×10^{-3})	2.1×10^{-1} (1.7×10^{-1})

^aBased on data from Greenblatt and Mirvish, 1973.

The statistical likelihood ratio test of Rai and Van Ryzin (1981) yields a chi-squared value of 1.08, which for one degree of freedom is not significant, even at 25% level. Therefore, the observed data provide no evidence to reject the one-hit model, and the data in the first row of Table 10-8 are believed to be relevant estimates of the doses for various risks.

The exact meaning of these estimates for humans is difficult to assess since the data from animals cannot be converted directly to humans, either as mg/kg body weight or as mg/cm² body surface, without assuming that humans are concurrently exposed to 6 g of piperazine per kilogram of diet. Such an assumption is unreasonable in view of the exposures of the general population to piperazine. Moreover, piperazine is nitrosated more readily than most common amines that are present in the diet (Greenblatt and Mirvish, 1973).

Therefore, the only conclusion that one can draw from this risk extrapolation is that increased levels of nitrite consumed in the presence of exceedingly high levels of a nitrosatable amine lead to a rather linear dose-response curve for nitrite. By extrapolating such a dose-response curve down to zero dose, it can be seen that a reduction in the frequency with which animals consume nitrite in the diet would lead to a proportional reduction in the incidence of lung adenomas. For example, halving the dose of nitrite would halve the frequency of animals with lung adenomas. Extrapolation and conversion techniques do not have the capability of determining the exact relevance of this observation to humans, with regard to the effect on in vivo formation of N-nitroso compounds resulting from reduced exposure to nitrite in the presence of nitrosatable amines.

Additional Estimates of Risk. In this section, the calculations of risk for N-nitroso compounds are combined with exposure to nitrate and nitrite from food and water (Table 5-20 and 5-21) and exogenous exposure from other sources (Table 8-4, as modified by using data for in-vivo formation, Table 8-3) to arrive at estimates of risk for certain defined subgroups of the population.

Underlying these calculations are the assumptions that nitrosamines are carcinogenic in humans, that NDMA is the main source of exposure for humans, that NDMA is representative of all nitrosamines (even though its potency in animals is greater than that of many other nitrosamines), and that NDMA is as carcinogenic in humans as it is in animals. The committee recognizes that these assumptions may not be valid. Certain alkyl nitrosoureas and other nitrosamides

are more carcinogenic than NDMA, and humans may be more sensitive to their effects than rodents, particularly if the exposure occurred during pre- or neonatal development.

Thus, the estimates were taken from Table 10-3, i.e., a lifetime risk of 10^{-6} is incurred by a person whose daily exposure to nitrosamines is 5.84 ng/kg body weight per day, if converted on a unit of body weight basis, and 0.85 ng/cm² body surface per day, if converted on a unit of body surface area basis.

In addition, to calculate the total amount of nitroso compounds formed endogenously, the committee assumed that amino substrates in the stomach are nitrosated at the same rate as proline and that the daily intake of amines in the diet is 4 g. The committee recognizes that weakly basic amines and amides are more readily nitrosated than proline and that the actual exposure to amines may be considerably higher or lower than 4 g/day.

Using these assumptions and estimates, the committee calculated the risk for seven groups with low to high levels of exposure (Table 10-9). Assuming linearity of the risk at low doses, the low-dose risk for a daily exposure dose (d in μ g) is calculated:

$$P(d) = 1,000 \times \theta \times d = (1.81 \times 10^{-5}) d,$$

where $\theta = P_0/d_0 = 10^{-6}/(0.85 \times 65)$ and 0.85×65 is the estimated daily dose of NDMA in ng for a person weighing 65 kg to yield a risk (P_0) of 10^{-6} over a lifetime (Table 10-3). Thus, the lifetime risk for Group 1 of Table 10-9 is estimated by the following calculation:

$$\begin{aligned} P(3.1) &= \frac{1,000 \times 10^{-6} \times 3.1}{0.85 \times 65} \\ &= (1.81 \times 10^{-5}) \times 3.1 = 5.6 \times 10^{-5}. \end{aligned}$$

Similar calculations were also made for the other six groups (Table 10-9).

If we assume that 130 million people in the United States

**Estimates of Lifetime Risk of Cancer for Various Population Groups
Based on Exogenous and In-Vivo Exposure to Nitrosamines.
Numbers in Parenthesis Indicate Reduced Exposure if All
Nitrite were Removed from Cured Meats**

Source of Exposure ^b	Exposure to Nitrosamines, $\mu\text{g}/\text{Person}/\text{Day}$ ^a						
	Group 1 Average Diet, Nonsmoker	Group 2 Average Diet, Smoker	Group 3 High Cured Meat Diet ^c	Group 4 Vegetarian, ^d No Beer	Group 5 Nitrate-Rich Water, ^e No Beer, Nonsmoker	Group 6 High Risk ^f	Group 7 Low Risk ^g
<u>Endogenous^h</u>							
Diet (Total)	1.3(1.1)	1.3(1.1)	2.0(1.1)	12.0(12.0)	14.0(13.0)	16.0(13.0)	1.3(1.1)
<u>Exogenous</u>							
Cosmetics	0.41(0.41)	0.41(0.41)	0.41(0.41)	0.41(0.41)	0.41(0.41)	0.82(0.82)	
Car Interiors	0.20(0.20)	0.20(0.20)	0.20(0.20)	0.20(0.20)	0.20(0.20)	0.50(0.50)	0.02(0.02)
Beer	0.97(0.97)	0.97(0.97)	0.97(0.97)	0	0	3.9(3.9)	0
Bacon	0.17(0)	0.17(0)	0.68(0)	0	0.17(0)	0.68(0)	0
Tobacco smoke	0	17(17)	0	0	0	35.0(35.0)	0
Work	0	0	0	0	0	250(250)	0
TOTAL EXPOSURE	3.1(2.7)	20.1(19.7)	4.3(2.7)	12.6(12.6)	14.8(13.6)	307(303)	1.3(1.1)
LIFETIME RISK ⁱ	5.6×10^{-5}	3.6×10^{-4}	7.8×10^{-5}	2.3×10^{-4}	2.7×10^{-4}	5.6×10^{-3}	2.3×10^{-5}
REDUCED LIFE- TIME RISK ^j	4.9×10^{-5}	3.5×10^{-4}	4.9×10^{-5}	2.3×10^{-4}	2.5×10^{-4}	5.5×10^{-3}	2.1×10^{-5}
RANGE OF LIFETIME RISK ^k	8.2×10^{-6} to 1.8×10^{-4}	5.3×10^{-5} to 1.2×10^{-3}	1.1×10^{-5} to 2.5×10^{-4}	3.4×10^{-5} to 7.4×10^{-4}	3.9×10^{-5} to 8.7×10^{-4}	8.2×10^{-4} to 1.8×10^{-2}	3.4×10^{-6} to 7.4×10^{-5}

^a Assuming that the average body weight is 65 kg.

^b Taken from Tables 8-3 and 8-4.

^c Assuming 4 times the average intake of meat and bacon.

^d Assuming 4 times the average intake of vegetables and no meat.

^e Assuming that 160 mg of nitrate is provided by consumption of nitrate-rich water.

^f Assuming 4 times the average intake of meat and bacon and the use of nitrate-rich water (which provides 160 mg of nitrate daily [see Table 5-20]), heavy cosmetic use (twice the average), frequent use of a new automobile, heavy beer consumption (4 times the average), heavy smoker (twice the average), and high occupational exposure.

^g Assuming an average diet, no cosmetics, light automobile use (15 min/day), no beer, no bacon, nonsmoker, and no occupational exposure.

^h Amount of nitrosoproline formed in vivo as a result of various types of exposure (see Table 8-3 and 8-4 for methods used to arrive at these estimates).

ⁱ Based on mg/cm^2 body surface area. These amounts should be divided by 6.85 to obtain an estimate for ng/kg body weight.

^j Assuming that all nitrite is removed from cured meats.

^k The higher extreme is derived by using the one-hit model, i.e., multiplying the lifetime risk by 3.2. The lower extreme is obtained by dividing by 6.85 the lifetime risk estimates calculated for units of body surface area (footnote i), to obtain the estimate for units of body weight.

This would lead to an effective rate of 1.9 (130/70) million people at risk per year.

The Possible Effect of Eliminating Nitrite from Cured Meats. The row under the lifetime risk in Table 10-9 provides rough estimates of reduced risk of cancer if nitrite were removed from cured meats. These estimates were derived from speculative calculations based on reduced exposures to nitrosamines that might be expected if all nitrite were removed from cured meats. If 130 million people in the United States are assumed to fall into Group 1, as before, the reduced number of deaths per year from cancer due to exposure to nitrosamines would be:

$$91 = \frac{130 \times 10^6 \times 4.9 \times 10^{-5}}{70}.$$

Therefore, for Group 1, the reduction of deaths per year due to removal of nitrite from cured meats would be approximately:

$$13 = (104 - 91).$$

Likewise, if we assume that another 50 million people belong to Group 2 (average diet and smoker), removal of nitrite would lead to a reduction of approximately 7.1 deaths per year due to tumors induced by exposure to nitrosamines:

$$7.1 = \frac{(50 \times 10^6)}{70} \times (3.6 - 3.5) \times 10^{-4}.$$

Assuming that another 20 million people in the United States belong to the high cured meat diet group (Group 3), 10 million to the high risk group (Group 6), and 10 million to the low risk group (Group 7), then the following reduction in deaths per year due to cancer from exposure to nitrosamines would result from the removal of nitrite from cured meats:

High Cured Meat Diet (Group 3):

$$8.3 = \frac{(20 \times 10^6)}{70} \times (7.8 - 4.9) \times 10^{-5}$$

High Risk (Group 6):

$$14.3 = \frac{(10 \times 10^6)}{70} \times (5.6 - 5.5) \times 10^{-3}$$

Low Risk (Group 7):

$$0.3 = \frac{(10 \times 10^6)}{70} \times (2.3 - 2.1) \times 10^{-5}$$

Adding the numbers of deaths estimated for Groups 1, 2, 3, 6, and 7 indicates that elimination of nitrite from cured meats would result in a reduction of approximately 43 deaths per year (i.e., $13 + 7.1 + 8.3 + 14.3 + 0.3 = 43$) from cancer if all cancers lead to death in the general mixed population of 130 million nonsmokers eating an average diet, 50 million smokers eating the same diet, 20 million eating a high cured meat diet, 10 million high risk individuals who are exposed occupationally as well as from other sources, and 10 million low risk individuals. This estimate is based on conversion of the dose to unit of surface area for humans. When expressed as unit of dose per unit of body weight, the resulting reduction in the number of deaths would be approximately 6.3.

The calculations given in Table 10-9 and those that follow the table are all presented as specific numbers. However, these numbers should only be considered as crude estimates of risk and that they represent a number based on the specific assumptions made. In general estimates over a range of values are more realistic and depend on the assumptions made. For example, if one uses the one-hit model estimate from Table 10-2 in developing Tables 10-3 and 10-9, the resulting estimates would be multiplied by a factor of 3.2 ($0.097 \div 0.03$). Thus, the lifetime risks from Group 1 would be 1.8×10^{-4} . Likewise, as pointed out in footnote i of Table 10-9, if the ng/kg body weight conversion were used the estimates in Table 10-9 should be divided by 6.85, yielding a lifetime risk for Group 1 of 8.2×10^{-6} . The last row of Table 10-9 shows this range for each population group using these two variations in the risk estimates.

Furthermore, it should be realized that all calculations in Table 10-9 and the reduction in the number of deaths assumed the mid-points of exposure given in Tables 5-20, 5-21, and Table 8-4 and that one could easily widen these ranges. However, the committee believes that the ranges given in Table 10-9 for various population groups and the estimated reduction in cancer deaths (6 to 138 per year) if nitrite were removed from cured meats, are plausible estimates based on the assumptions cited herein.

Another point that must be realized is that the above estimates completely ignore subpopulations that are heterogeneous and that may be more sensitive to the carcinogenic effects of nitrosamines because of a genetic or metabolic abnormality or other condition. If such populations exist, reduced exposure to nitrite for these populations might considerably reduce these risks.

Discussion and Summary

The speculative risk estimates made by the committee indicate that from approximately 6 to 138 cases of cancer could be avoided in the United States annually if nitrite were removed from cured meats. This calculation and conclusion are very speculative since they are based on the following assumptions: (1) that humans are as susceptible as rats to cancer induction by all nitrosamines, including NDMA; (2) that NDMA is the main source of exposure for humans and is, therefore, representative of all nitrosamines; (3) that the potency and low-dose linear behavior of all nitrosamines are similar to those of NDMA; (4) that all nitrosatable amines behave like proline in the stomach; (5) that the exposure levels are those given in Tables 5-20, 5-21, 8-3, and 8-4; and (6) that the population consists of 130 million from Group 1 (the average nonsmoker), 50 million from Group 2 (smokers), 20 million from Group 3 (high cured meat diet), and 10 million each from Groups 6 and 7 (high and low risk). The last assumption, concerning population mix, is not too crucial because among the seven groups, Group 1, the largest group, has the second lowest ratio of lifetime risk to reduced lifetime risk (0.9), as shown in Table 10-9. The lowest ratio is for the high cured meat diet group (0.6).

From these calculations and speculations, it appears that a large reduction in exposure to nitrosamines in work environments, from cigarette smoke, and possibly from certain cosmetics and drugs would have a greater life-saving effect than the removal of nitrite from cured meats. The main reason for this is that exposure to nitrosamines

To the degree that one places confidence in the estimate made by the Food and Drug Administration Task Force (Food and Drug Administration, 1979b) that 22 deaths from butulism would result from the omission of nitrite in meat products, that number could be compared to the possibility of 6 to 138 deaths estimated above. The committee does not believe that such a comparison is advisable since both numbers are rough estimates.

OVERALL SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Evidence of carcinogenicity provided by well-conducted experiments in animals should be regarded as indicating a potential for carcinogenicity in humans. This is especially true when results of investigations have demonstrated carcinogenicity in more than one species. There are no completely reliable methods for using data obtained from animal experiments to derive the magnitude of tissue- or organ-specific carcinogenic potency of a chemical in humans.

Based on one experiment in rats and assuming low dose linearity, the committee has estimated that the lifetime risk of cancer would be one in a million, if humans were exposed to a daily dose of 5.8 to 19 ng of nitrosodimethylamine per kilogram of body weight or 0.85 to 2.7 ng of nitrosodimethylamine per cm^2 of body surface. In arriving at this estimate, the committee has also assumed that (1) the dietary doses given to rats can be converted to unit of dose per unit of body weight or per unit of body surface area to reflect human exposure and (2) that nitrosodimethylamine is the main source of exposure to nitrosamines for humans and is therefore representative of all nitrosamines, even though its potency in animals is greater than that of many other nitrosamines.

The committee also examined seven hypothetical population groups and estimated that the lifetime risk of cancer from exposure to all sources of nitrosamines would be 820 to 18,000 per million for a high risk group (including occupational exposure), 11 to 250 in a million for a high cured meat diet group, 8 to 180 in a million for an average population of nonsmokers, and 3 to 74 in a million for a low risk group.

The committee wishes to emphasize that the validity of these estimates of risk is limited by some significant gaps in our knowledge: insufficient data about the average and extreme levels of exposure to nitrate, nitrite, and N-nitroso compounds, multiplicity of inadequately characterized variables that determine the extent of endogenous nitrosation, uncertainty about the molecular mechanisms leading to the carcinogenic effect of N-nitroso compounds and their precursors; uncertainty about the comparable ability of humans and laboratory animals to repair

introduce additional uncertainty. Therefore, the committee suggests that the numerical estimates be used solely as rough indicators of the relative risk to each of these population groups. The absolute numbers stated in this chapter are not intended as a guide for policy formation, nor should they be understood by the public to be final and definitive.

Although a reduction in exposure to nitrite is likely to reduce the risk of cancer, there is insufficient evidence to support the plausible assumption that a reduced exposure to nitrate and nitrite will lead to a directly proportional reduction in the risk to human health. There is better evidence for N-nitroso compounds: Studies of nitrosodimethylamine in animals indicate that a directly proportional reduction in risk could result from the reduction of exposure to N-nitroso compounds.

Nitrosamines formed endogenously from nitrite in cured meats provide only a small proportion of the total exposure of the general population to nitrosamines from all sources. Thus, it does not appear that the reduction of nitrite in cured meats will lead to a major decrease in risk to humans arising from total exposure to nitrosamines. However, if only dietary contributors to exposure to N-nitroso compounds are considered, the diminution in risk will be proportionally greater if nitrite were removed from cured meats. These conclusions are based on average exposure to N-nitroso compounds and do not fully consider the effects of exposure to peak levels, which may be a critical factor.

The committee examined various approaches to estimating the risk of botulism and the increment in its incidence that might arise from omitting nitrite from cured products. It found that previous attempts to derive such estimates were based on speculation with which it did not wholly concur. It concluded that a more adequate data base must be developed before one can predict the likelihood of a product becoming toxic and, from this, the incidence of botulism. However, the committee believes that the degree of protection against botulism is likely to decrease if the essential preservative uses of nitrite are substantially reduced without introducing an efficacious, but safer alternative.

In the interest of defining the absolute risk of botulism and the increment in this risk if the use of nitrite were diminished, the committee recommends that investigations be pursued to determine the incidence of and resulting mortality from botulism.

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The health effects
nitrate, nitrite,
nitroso compounds

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